IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Schwarz et al. Application No.: 09/424,498 Filed: February 15, 2000 For: PHARMACEUTICAL PREPARATION COMPRISING VWF PROPEPTIDE Customer No.: 20350	Confirmation No. Examiner: Schnizer, Holly G. Technology Center/Art Unit: 1653 DECLARATION OF PETER TURECEK UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Peter Turecek, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

2. All statements herein made of my own knowledge are true, and statements ma de on information or belief are believed to be true and correct.

3. I am a named inventor on the above-referenced patent application. I received my B.S. from University of Vienna in 1985 and my Ph.D. from the University of Vienna in 1987. I am currently the Director of Global Preclinical R & D at Baxter BioScience. I have been in this and related positions for 16 years. I have been in this position since 1991. I have numerous scientific publications in the field of plasma proteins, blood coagulation, hemostasis, immunology, plasma fractionation technology, including pathogen inactivation methods.. A copy of my CV is attached as Exhibit 1.

4. I have read and am familiar with the contents of the subject patent application. I have also read the Office Action received from the United States Patent and Trademark Office dated October 5, 2004. It is my understanding that the Examiner is concerned that the claimed pharmaceutical preparations comprising at least 10 nM Willebrand Factor (vWF) propeptide ("pvWF") treated for virus inactivation and/or virus removal and suitable for therapeutic administration, are anticipated by the disclosure of Takagi *et al.*, *J. Biol. Chem.* 264(11): 6017-6020 (1989). More particularly, the Examiner has alleged that there is no basis to believe that the ppvWF preparation of Takagi *et al.* is contaminated with viruses.

5. This declaration is presented to demonstrate that there is a scientific basis to believe that the ppvWF preparation of Takagi *et al.* has a serious medical risk of being contaminated with viruses, *i.e.*, that there is a scientific basis to believe that the ppvWF preparation of Takagi *et al.* is contaminated with viruses.

6. It is well settled in the art that there is a serious medical risk that blood and blood products are contaminated with viruses, and that viral inactivation procedures are required to render blood and blood products suitable for therapeutic use. Numerous scientific references discuss the issue of viral contamination in blood products and emphasize the importance of viral elimination and inactivation procedures to minimize the risk of viral contamination of blood products, *i.e.*, so that the blood products are suitable for therapeutic administration (*see, e.g.*, Remington *et al.*, *Vox Sang.* 87(1):10-8 (2004); Jackson, *Br. J. Biomed. Sci.*, 60(4):227-32 (2003); and European Agency for the Evaluation of Medicinal Products (EMEA), *Note for Guidance on Assessing the Risk for Virus Transmission - New Chapter 6 of the Note for Guidance on Plasma-derived Medicinal Products* CPMP/BWP/5180/3 (2003), copies enclosed as Exhibits, 2, 3, and 4). Jackson *et al.* explains that blood can harbor pathogens, including viruses (*see*, page 227, col. 2, first paragraph) and points out that "viruses are the main target for pathogen activation" in blood products such as plasma and platelet concentrates (*see, e.g.*, page 228, col. 2, fourth full paragraph and page 229, col. 2, ¶¶ 6 and 7). As the EMEA states, "it should be assumed that each virus particle entering the plasma pool might be infectious" (*see*, page 3, §2, second paragraph of Exhibit 4). Jackson stresses the importance of using viral elimination or inactivation steps in the preparation of any blood products because "effective pathogen inactivation technique[s] would achieve greater transfusion safety" (*see*, page 228, col.

1, last full paragraph). Without such treatment, the blood products cannot be said to suitable for therapeutic administration. Jackson and Remington *et al.* also set forth the commonly used methods for viral inactivation of blood products: pasteurization, photochemical treatment, methylene blue treatment, solvent detergent treatment, and caprylate treatment. Moreover, both the EMEA and the World Health Organization ("WHO") have published materials to provide advice to ensure the safety for plasma derived medicinal products, *i.e.*, by minimizing the risk of virus transmission by plasma-derived products (copies enclosed as Exhibits 5, and 6). Both the EMEA and the WHO advise the inclusion of virus removal and inactivation steps during the manufacture of blood products for therapeutic administration to minimize the risk of viral contamination (*see, e.g.*, page 2, §§ 1.5(i)-(iii) of Exhibit 5, and page 2, col. 1, second paragraph of Exhibit 6). Therefore, based on what is known in the art, one of skill in the art would appreciate that unless blood products are subjected to viral elimination or inactivation procedures, blood products are not suitable for therapeutic administration.

7. Takagi *et al.* describes preparation of ppvWF from platelet concentrates using column chromatography. The ppvWF preparation of Takagi *et al.* does not undergo any procedures for viral inactivation or elimination.

(a) As set forth in Takagi *et al.*, platelet concentrates are first sonicated, then dialyzed (*see*, page 6017, col. 2, lines 20-26). Neither sonication nor dialysis inactivates or removes any viruses present in the platelet concentrates. It is well known that intact viruses can be prepared from sonicated cells. It is also well known that dialysis is not effective in removing viruses from aqueous solutions. Dialysis is commonly used for removing salt from aqueous solutions while retaining a product of interest (*e.g.*, a protein). The small size difference between protein products of interst (*e.g.* ppvWF) and viruses requires the use of dialysis membranes with pore sizes that do not allow the passage of viruses out of the solution. Therefore, the sonicated, dialyzed platelet concentrates will contain any viruses present in the initial platelet concentrate.

(b) According to the methods of Takagi *et al.*, the sonicated, dialyzed concentrates are next passed through a collagen column to capture collagen-binding proteins (*e.g.*, blood coagulation factors such as ppvWF) which are eluted by raising the salt concentration of the column buffer (*see*, page 6017, col. 2, lines 25-32). Many viruses bind collagen. Therefore, any viruses with collagen-binding proteins that are present in the platelet concentrates will bind to the collagen column and will be present in the eluate.

(c) Next, Takagi *et al.* pass the eluate from the collagen column through an organomercurial-agarose column which binds and eliminates only proteins with free SH groups (*see*, page 6017, col. 2, lines 32-36). With a few notable exceptions (*e.g.*, Factor XIII), intact proteins typically do not contain free SH groups. Free SH groups are extremely reactive and are usually present only in denatured proteins, degraded proteins, or proteins treated to expose SH groups. Thus, viruses with intact proteins will also pass through the column and be present in the eluate.

(d) Finally, Takagi *et al.* pass the eluate from the organomercurial-agarose column though a lectin-agarose column to capture glycoproteins which are then eluted using α-methylmannose and the eluate containing glycoproteins is dialyzed (*see*, page 6017, col. 2, lines 36-39). Many viruses have glycosylated proteins (*e.g.*, HIV gp160) and such viruses will also be present in the eluate. As discussed above, dialysis does not remove viruses from aqueous solutions. Thus, the final eluate will contain glycoproteins including, ppvWF and viruses with glycosylated proteins and/or collagen-binding proteins.

(e) Therefore, in the absence of any viral elimination or activation steps, there is a serious medical risk that viruses present in the platelet concentrates would also contaminate the final ppvWF preparation of Takagi *et al.* Moreover, Takagi *et al.* does not describe ***any*** of the standard viral inactivation or elimination procedures (*i.e.*, pasteurization, photochemical treatment, methylene blue treatment, solvent detergent treatment, and caprylate treatment), much less that they can be used to remove or eliminate viruses. Accordingly, Takagi *et al.*, does not describe the presently claimed preparation of pro-vWF that is suitable for therapeutic administration.

8. The distinguishing feature between the presently claimed pharmaceutical preparation of provWF and the ppvWF preparation described in Takagi *et al.* is the striking difference in the risk of viral contamination. The significantly improved safety of the claimed pro-vWF preparations is vitally important to patients receiving the preparations. Therefore, the crucial issue is not whether the ppvWF preparation of Takagi *et al.* does indeed contain viruses, but rather, whether it could be contaminated with viruses. From a medical point of view, there is a serious risk of contamination associated with the ppvWF preparation of Takagi *et al.* As discussed above, due to the high medical risk of contamination, viral inactivation procedures are essential for products obtained from plasma. Thus, a skilled person, *i.e.*, a physician, would not

consider the ppvWF preparation of Takagi *et al.* to be suitable for therapeutic administration. The high medical risk is minimized in the claimed pro-vWF preparations by viral inactivation procedures. In contrast, the ppvWF preparation described in Takagi *et al.* was not treated with any viral inactivation methods and is not suitable for therapeutic administration.

9. In view of the foregoing, it is my scientific opinion there is a scientific basis to believe that the ppvWF preparation of Takagi *et al.* has a serious risk of being contaminated with viruses and, accordingly, the ppvWF preparation is not suitable for therapeutic administration.

10. The Declarant has nothing further to say.

Date: 2005-04-04

By: _____

Peter Turecek, Ph.D.

CURRICULUM VITAE

Name: Peter L. TURECEK, PhD

Adress: (business)
BAXTER AG
Industriestraße 67
A-1221 Vienna
+43-1-20100-2520
peter_turecek@baxter.com

Address: (private)
Hauptstraße 59g
A-3400 Klosterneuburg
+43-2243-31177
peter.turecek@univie.ac.at

Date of birth: May 9th, 1961
Place of birth: Vienna, Austria
Citizenship: Austria
Marital status: married, 3 children

Academic education:

1980 - 1985	Pharmacy, University of Vienna Degree: Magister pharmaciae 1985
1985 - 1987	Biochemistry, University of Vienna Thesis, Degree: Doctor rerum naturalium (Ph.D.) 1987
1980 - 1988	Business Administration, University of Economics, Vienna, Diploma 1988

Other training:

Protein chemistry, chromatography, process validation, hematology, coagulation, immunology

Specific fields of expertise:

Protein purification and characterization, plasma fractionation, virus inactivation, molecular biology, recombinant proteins, coagulation physiology, biochemical and analytical techniques, diagnostics, pharmaceutical biotechnology, pharmacology and toxicology

Professional experience:

present	Director Global Preclinical R&D and Product Development BioScience Division, Baxter Healthcare, BAXTER AG, Vienna
2000 – 2001	Member Diagnostics Management Team, Research & Development Therapeutic Proteins Hyland Immuno Division, Baxter Healthcare, BAXTER AG, Vienna
1998 – date	Associate Professor of Pharmacology (Habilitation) University of Vienna
1997 - 1999	Head, Depts. Product & Process Development Coagulation and Pharmaceutical Technology, Global R&D Plasma, BAXTER/HYLAND-IMMUNO, Vienna
1996 - 1999	Head, Dept. of Process Monitoring Coagulation, Production Blood-Plasma Derivatives, Österreichisches Institut für Hämoderivate GesmbH, Vienna
1996 - date	Legal Permission for Animal Experiments (Tierversuchsleiter gem. Österr. Tierversuchsgesetz 1988)

1992 - 1995	Head, Dept. of Biochemical Research on Haemostasis and Thrombolysis, IMMUNO AG, Vienna
1991 - 1992	Deputy Head, Dept. of Biochemical Research on Coagulation and Fibrinolysis, IMMUNO AG, Vienna
1991	Head, Laboratory of Recombinant Blood Factors, Biomedical Research Center, IMMUNO AG, Orth
1989 - 1990	Research Assistant, Dept. of Microbiology and Molecular Biology, Biomedical Research Center, IMMUNO AG, Orth
1988 - 1989	Assistant Professor, Institute of Biochemistry, University of Vienna
1986 - 1989	Consultant for R&D Projects for MAGINDAG, Steirische Magnesit Industrie AG, Vienna and Research Biotechnology, Chemie Holding AG, Linz
1985 - 1988	Technician, Institute of Biochemistry, University of Vienna
1982	Laboratory Assistant, Dept. of Radiopharmaceuticals, Institute of Chemistry, Österreichisches Forschungszentrum, Seibersdorf
1980	Laboratory Assistant, Polymer Laboratory, Reichhold Chemie GmbH., Vienna

Technical and Scientific Expert in front of National and International Authorities (since 1992):

- European Patent-Office
- US Patent and Trademark Office
- German Patent-Office
- Austrian Patent-Office
- Paul-Ehrlich-Institute, Germany
- Medicines and Healthcare products Regulatory Agency (MHRA) – United Kingdom
- DIN Deutsches Institut für Normung e. V. NAMed AA C 5 - Arbeitsausschuss Hämatologie
- EDQM-European Pharmacopoeia Commission – Permanent Specialist Group of Experts Nr. 6B on Human Blood and Blood Products (since 2003)
- European Agency for the Evaluation of Medicinal Products (EMEA)
- US Department of Health and Human Services: Food and Drug Administration (FDA)
- European Commission, Directorate-General XII Science, Research and Development Group of Experts on Demonstration in Life Sciences (1998)

Teaching Experience:

- Lectureship at the University of Vienna in Pharmacology and Toxicology (since 1998):
- Lectures on Pharmaceutical Biotechnology,
- Lectures for Diploma and PhD-Students,
- Course on Pharmacology, Pharmacotherapy, Toxicology and Bromatology III (2002-2003)
- Lectureship Entrepreneurship MBA Applied Biomedicine, Donau-University Krems (2002)
- Lectureship Professional MBA Biotech Management, Donau-University Krems (2003)
- Lectureship in Pharmacology, FH-Studiengang „Medical and Pharmaceutical Biotechnology", University of Applied Management Sciences, Fachhochschule Krems (since 2003)
- Lectureship FH-Studiengang „Biotechnologie", FH-Campus Wien (2003)

Honorary Positions and Memberships:

- Council, "Höhere Bundes-Lehr- und Versuchsanstalt für chemische Industrie", Vienna XVII, Rosensteingasse (1998 - 2002)
- Development Team FH-Studiengang Medical and Pharmaceutical Biotechnology, International Management Center, Fachhochschule Krems (2001-2002)
- „Wirtschaftsboard", University of Applied Management Sciences, Fachhochschule Krems (since 2003)
- Austrian Biochemical Society
- Austrian Society of Biotechnology
- Austrian Pharmaceutical Society
- World Federation of Haemophilia
- The Protein Society
- The International Society on Thrombosis and Haemostasis

Ad hoc Reviewer:

- Thrombosis and Haemostasis
- Thrombosis Research
- Journal of Thrombosis and Haemostasis
- Blood Coagulation Fibrinolysis
- Blood

Grants and Awards:

- Research Grant "Jubiläumsfonds der Österr. Nationalbank" (1987-1988)
- 12 Baxter Technical Awards 1998 (Categories: Distinguished Corporate Contribution, Special Accomplishment)
- 7 Baxter Technical Awards 1999 (Categories: Customer First, Special Accomplishment)
- 6 Baxter Technical Awards 2000 (Categories: Customer First, Special Accomplishment, Outstanding Corporate Achievement)
- Baxter Technical Award 2003 (Category: Outstanding Innovation)

Number of Publications: 88 papers and book articles
203 lectures and abstracts

Number of Granted, Published or Filed Patents: 411 (45 patent families)

Vox Sanguinis (2004) **87**, 10–18

ORIGINAL PAPER

Inactivation of West Nile virus, vaccinia virus and viral surrogates for relevant and emergent viral pathogens in plasma-derived products

K. M. Remington,[1] S. R. Trejo, G. Buczynski, H. Li, W. P. Osheroff, J. P. Brown, H. Renfrow, R. Reynolds & D. Y. Pifat

Preclinical Research and Pathogen Safety, Biological Products Division, Bayer Health Care, LLC, Research Triangle Park, NC, USA

Vox Sanguinis

Background and Objectives Human plasma is the source of a wide variety of therapeutic proteins, yet it is also a potential source of viral contamination. Recent outbreaks of emergent viral pathogens, such as West Nile virus, and the use of live vaccinia virus as a vaccine have prompted a reassessment of the viral safety of plasma-derived products. The purpose of this study was to evaluate the efficacy of current viral inactivation methods for West Nile and vaccinia viruses and to reassess the use of model viruses to predict inactivation of similar viral pathogens.

Materials and Methods Virus-spiked product intermediates were processed using a downscaled representation of various manufacturing procedures. Virus infectivity was measured before and after processing to determine virus inactivation.

Results The results demonstrated effective inactivation of West Nile virus, vaccinia virus and a model virus, bovine viral diarrhoea virus, during pasteurization, solvent/detergent treatment and caprylate treatment. Caprylate provided rapid and effective inactivation of West Nile virus, vaccinia virus, duck hepatitis B virus and Sindbis virus. Inactivation of West Nile virus was similar to that of bovine viral diarrhoea virus.

Conclusions This study demonstrates that procedures used to inactivate enveloped viruses in manufacturing processes can achieve inactivation of West Nile virus and vaccinia virus. In addition, the data support the use of model viruses to predict the inactivation of similar emergent viral pathogens.

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Key words: caprylate, pasteurization, solvent/detergent, vaccinia virus, virus inactivation, West Nile virus.

Introduction

Plasma-derived products, such as clotting factors, intravenous immunoglobulins, alpha$_1$-proteinase inhibitor (α_1-PI) and human serum albumin, are routinely used in clinical practice and the safety of these products with respect to transmission of infectious viruses has never been higher. The high margin of viral safety can be attributed to a number of measures that manufacturers have implemented during the last decade. These measures are part of a multifaceted strategy for pathogen safety that includes donor screening, donation testing, virus inactivation or removal measures during manufacture, strict adherence to good manufacturing procedures and post-use surveillance of products. However, recent outbreaks of emergent viruses, such as West Nile virus (WNV), severe acute respiratory syndrome (SARS)-associated coronavirus and monkeypox, have indicated that potential threats to the blood supply exist and have resulted in a re-evaluation of the current pathogen safety strategy for plasma products.

Although donor deferral and plasma donation screening effectively provide safety for known pathogens, the presence of a new or emergent virus cannot be detected when its existence is not anticipated. Consequently, robust viral clearance

Correspondence: Kathryn M. Remington, Cardinal Health, P.O. Box 13341, Research Triangle Park, NC 27709, USA
E-mail: kathryn.remington@cardinal.com

procedures within manufacturing processes are critical. The capacity of these procedures to remove or inactivate viruses is validated in small-scale studies in which high levels of virus are spiked into a manufacturing intermediate and the reduction of infectious virus is measured. Typically, a number of these experiments are performed, each using a different virus that could potentially be present in plasma. Some potential contaminants, such as hepatitis C virus (HCV) and parvovirus B19, cannot be readily cultured *in vitro*. For viruses like these, a closely related virus with similar physicochemical properties, and which can be easily propagated in the laboratory, is used [1]. Like HCV, bovine viral diarrhoea virus (BVDV) is a member of the Flaviviridae and has been used for many years as a model for HCV. Other viruses, such as the Flaviviruses yellow fever virus and tick-borne encephalitis virus, as well as the Alphaviruses Sindbis virus and Semliki Forest virus, have also been used to model HCV. Inactivation/removal of BVDV and other HCV model viruses by various manufacturing processes has effectively represented HCV clearance, as evidenced by the lack of HCV transmission by products which have claimed significant levels of BVDV clearance.

WNV, a mosquito-borne encephalitis virus, is now epidemic in the United States. Most WNV-infected individuals have subclinical infections, with a short, but defined, viraemic period. During epidemics of WNV infection, when the prevalence of viraemia within the population is high, the risk of a viraemic blood or plasma donor is also high. In fact, in 2003, WNV was transmitted to transplant recipients from an infected organ donor and to recipients of whole blood and platelets from infected donors [2]. This resulted in the implementation of a United States Food and Drug Administration (FDA)-endorsed WNV screening programme for whole blood. The programme includes donor deferral questions and nucleic acid amplification tests (NATs) [3–5]. The FDA and the European Agency for the Evaluation of Medicinal Products (EMEA) concur with manufacturers of plasma products that various viral-inactivation measures used during the processing of these products are sufficient to provide a high degree of safety for this virus [3,6].

The live vaccinia virus (VV) vaccine has recently been used as part of the United States National Smallpox Immunization Program. In an interim recommendation, the Centers for Disease Control advised immunization with this vaccine for individuals at risk for monkeypox infection [7]. The possibility of transmission of VV during whole blood or plasma donations by recently vaccinated individuals has resulted in recommendations by the FDA to defer donors who have recently received the smallpox vaccine [8].

We have recently evaluated the inactivation of two newly emergent potential contamination threats: WNV and VV. VV is a unique enveloped virus, with complex lipid membrane structures [9]. Although some studies have evaluated VV inactivation, it is not always included in the panel of viruses used for virus clearance studies for plasma-derived products. WNV, a member of the Flaviviridae, is closely related to BVDV, which has been used for many years as a model for HCV. It would be expected, then, that WNV inactivation would be similar to that of BVDV, and that WNV-inactivation studies could verify the validity of using BVDV as a model for virus-inactivation studies. The virus-inactivation data presented here demonstrate that current virus-inactivation procedures provide inactivation of WNV and VV and support the use of virus-inactivation data from model viruses.

Materials and methods

Plasma product intermediates

Manufacturing process intermediates for anti-haemophilic factor (AHF) (Koate®-DVI), intravenous immunoglobulin (IVIG-S/D when produced using the solvent/detergent process: Gamimune®N; IVIG-C when produced using the caprylate/chromatography process: Gamunex®), intramuscular immunoglobulin (IGIM) (BayGam®), α_1-PI (Prolastin®) and albumin (5% and 25%; Plasbumin®) were obtained from the Bayer HealthCare plasma fractionation facility (Clayton, NC). Human plasma protein solution (HPPS) was obtained from the Bayer HealthCare recombinant factor VIII (Kogenate®FS) manufacturing facility (Berkeley, CA). HPPS is used as a component of the production medium for Kogenate®FS and is a 5% human albumin protein solution.

Pasteurization

For pasteurization studies, an aliquot of stabilized α_1-PI, HPPS, 5% albumin or 25% albumin was heated to 60 ± 0·5 °C and then spiked to 10% (v/v) with WNV, VV or BVDV. The virus spike and product were mixed well and incubated at 60 ± 0·5 °C for 10 h. Aliquots were removed at the indicated times, placed on ice and immediately assayed for infectious virus. A separate, unheated, virus-spiked product sample was used to determine the initial virus concentration. The α_1-PI intermediate, pH 6·5, was stabilized during pasteurization with 0·38 M citrate and 37% sucrose. HPPS and albumin were aqueous solutions at pH 7·0; HPPS and 5% albumin were stabilized with 4 mM *N*-acetyl-DL-tryptophan and 4 mM sodium caprylate, while 25% albumin was stabilized with 20 mM *N*-acetyl-DL-tryptophan and 20 mM sodium caprylate. Small-scale pasteurization experiments were performed in 30–50 ml volumes. During manufacture, albumin is pasteurized in its final container, and so the volume of the virus experiments was similar to that of production. Prolastin pasteurization experiments were approximately 0·04% of production scale and HPPS pasteurization experiments were 0·003% of production scale.

Tri-(*n*-butyl)-phosphate/Tween 80 treatment

During the production of AHF, the product intermediate is treated with 0·3% tri-(*n*-butyl)-phosphate (TNBP)/1% polysorbate 80 (Tween 80) for 6 h at 24–30 °C. For these studies, a concentrated stock solution of TNBP/Tween 80 was added to aliquots of AHF intermediate solution to 0·3%/1% or 0·15%/0·5%. Virus was added to the solution to 10% (v/v). The mixture was incubated at 28 ± 0·5 °C for 6 h, and aliquots were removed for titration at the indicated time-points. Virus, added to Hank's balanced salt solution (HBSS) to 10% (v/v), was an untreated control. Small-scale experiments were conducted in 40-ml volumes, approximately 0·5% of production scale.

Tri-(*n*-butyl)-phosphate/cholate treatment

During the production of IVIG-S/D or IMIG, the process intermediate is incubated in 0·3% TNBP/0·2% sodium cholate (cholate) for 6–8 h at 30 ± 2 °C. The protein concentration is adjusted to 6–7·5% and the pH is adjusted to 5·5–5·7. For VV-, WNV- or BVDV-inactivation studies, concentrated TNBP/cholate from a stock solution was added to an aliquot of process intermediate to 0·3%/0·2% or to 0·15%/0·1% and then spiked to 10% (v/v) with virus. The mixture was incubated at 28 ± 0·5 °C for 6 h and aliquots were removed at the indicated time-points for quantification of infectious virus. HBSS was spiked to 10% (v/v) with virus and used as an untreated control. Small-scale experiments were conducted in 40-ml volumes, approximately 0·005% of production scale.

Caprylate treatment

The caprylate-inactivation experiments were carried out as described previously [10]. Briefly, caprylate was added to Gamunex® intermediate to a specified final concentration. Throughout the addition of caprylate, the pH of the process intermediate was maintained at 5·1 by adding either 1·0 M acetic acid or 1·0 M sodium hydroxide. The solution was then spiked to 10% (v/v) with virus that had been pH-adjusted to 5·0–5·2 and the virus-spiked solution was incubated at 24 °C for 60 min. Aliquots for virus titration were removed from all solutions at the indicated times throughout the incubation. Small-scale experiments were conducted in 40-ml volumes, approximately 0·001% of production scale.

Although 20 mM caprylate is used in this step in production, the concentrations of caprylate evaluated in these studies ranged from 11 mM to 20 mM. As the Gamunex® intermediate contains ≈ 8 mM residual caprylate [11], caprylate from a 2·1 M stock solution was added to increase caprylate levels to within the desired range. To confirm that the targeted caprylate range was reached during inactivation experiments, the concentration of caprylate in the process intermediate following addition was measured. Analytical testing could not be performed on virus-spiked solutions, so mock-spiked solutions, containing 10% (v/v) virus propagation medium instead of virus, were generated.

Low-pH incubation

During the manufacture of Gamunex®, the final product at pH 4·0–4·3 is incubated at 23–27 °C for 21–28 days as an enveloped virus-inactivation step. For this study, the Gamunex® final product that had been adjusted to pH 4·4 was spiked to 10% (v/v) with WNV and incubated at 23 °C. Samples were removed on days 3, 7, 14 and 21, and infectious WNV was quantified. As pH 4·4 is above the normal pH range used in production, this represented a 'worst-case' situation. The volumes used for the virus-inactivation experiments were similar to the final container sizes of product.

Preparation and quantification of viruses

VV (the WR strain) was propagated and assayed at AppTec Laboratory Services (Camden, NJ). All WNV, except for that used in the Gamunex® low-pH incubation study, was also propagated and assayed at AppTec Laboratory Services, where infectious WNV or VV was quantified by making end-point serial log dilutions of the test samples or positive controls in serum-free medium. VV was assayed with a plaque assay using monkey kidney (BS-C-1) indicator cells. WNV (the NY-99 strain) was assayed with a plaque assay using African green monkey kidney (Vero) cells. For both viruses, 0·5-ml aliquots of serial dilutions of samples were plated on multiple wells of six-well plates. Virus titres were reported as plaque-forming units (PFU) per ml.

WNV (NY-99 strain), used in the Gamunex® low-pH incubation study, was propagated and assayed in Vero cells that were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Sindbis virus (Ar-339 strain) was propagated and assayed in Baby Hamster Kidney (BHK-21) cells obtained from the ATCC. BVDV (Kentucky-22 strain), obtained from the Biological Research Facility and Faculty (Ijamsville, MD), was propagated in Madin-Darby bovine kidney (MDBK) cells and assayed in bovine turbinate (BT) cells. Test samples were neutralized before they were assayed. All in-house viruses were quantified by tissue culture infectious dose 50 ($TCID_{50}$) and, for each dilution, 8–12 replicates of a 0·1-ml aliquot were plated in each well of 96-well microtitre plates. Cell monolayers were then observed microscopically for the presence of viral infection, as indicated by a viral cytopathic effect (CPE). Each well was scored as positive or negative, and the results were converted into a titre (log median $TCID_{50}$/ml) by using the method of Spearman & Karber [12,13].

Duck hepatitis B virus (DHBV), a hepatitis B virus (HBV) surrogate, was obtained from Hepadnavirus Testing, Inc. (Palo

Alto, CA) and consisted of DHBV-containing serum obtained from congenitally infected ducklings. The virus was assayed at AppTec Laboratory Services in primary duck hepatocytes. Samples were serially diluted and 0·5 ml of each dilution was assayed in quadruplicate in 48-well plates. Ten days following inoculation, monolayers were fixed and wells containing infected hepatocytes were identified using fluorescence microscopy after incubation with a murine monoclonal antibody to the DHBV surface antigen, washing and a subsequent incubation with a fluorescein isothiocyanate (FITC)-conjugated sheep antibody to mouse immunoglobulin G. Results were converted into a titre by using the method of Spearman & Karber [12,13].

For all virus assays, limits of detection were based on the results of cytotoxicity and viral interference experiments, which evaluated whether the product intermediates were toxic to the indicator cells or interfered with the ability of the virus to infect the cells.

Results

Pasteurization

The data in Table 1 show the inactivation of WNV, VV and BVDV in α_1-PI during a 10-h pasteurization at 60 °C. The protein, at a concentration of 25 mg/ml, is in a citrate buffer containing 0·38 M citrate and 37% sucrose for stabilization during heating. VV was reduced by 2 $\log_{10}$ within 1 h, and by 3 h was undetectable. WNV and BVDV were inactivated to the lower limits of detection within 3 h, and infectious virus was undetectable after 5 h at 60 °C. A total of ≥ 6·5 $\log_{10}$, ≥ 5·0 $\log_{10}$ and ≥ 4·9 $\log_{10}$ of WNV, VV and BVDV, respectively, were inactivated.

Table 1 Virus inactivation during pasteurization (10 h at 60 °C) of the alpha$_1$-proteinase inhibitor (α_1-PI)

Time at 60 °C (h)	WNV[a]	VV[a]	BVDV[b]
Unheated[c]	7·5	6·0	5·6
1	1·9	3·7	3·2
3	1·1	≤ 1·0	0·9
5	≤ 1·0	≤ 1·0	≤ 0·7
10	≤ 1·2	≤ 1·0	≤ 0·7
$\log_{10}$ reduction factor	≥ 6·5[d]	≥ 5·0	≥ 4·9

[a]Data from the West Nile virus (WNV) and vaccinia virus (VV) samples are expressed as $\log_{10}$ plaque-forming units/ml.
[b]Data from the bovine viral diarrhoea virus (BVDV) samples are expressed as $\log_{10}$ tissue culture infectious dose 50/ml.
[c]Virus was spiked into samples once they reached 60 °C. As the potential existed for immediate inactivation, the initial virus concentration was determined from an unheated sample.
[d]Inactivation was calculated from the value from the first time-point where samples were at the lower limit of detection. The increased detection limit for the WNV 10-h time-point was the result of reduced volume testing. WNV values represent the mean of three determinations, BVDV values represent the mean of two determinations and VV values represent a single determination.

WNV and VV were also inactivated during the pasteurization of HPPS (Table 2). This solution contained 5 mg/ml protein and was stabilized with 4 mM sodium caprylate and 4 mM acetyltryptophan. Complete inactivation of WNV was observed after 30 min of heating at 60 °C, resulting in a total inactivation of ≥ 7·1 $\log_{10}$. Approximately four $\log_{10}$ of VV was inactivated within 30 min; within 2 h of pasteurization, no infectious VV was detectable. A total inactivation of ≥ 5·7 $\log_{10}$ VV was observed.

Table 2 Viral inactivation during pasteurization (10 h at 60 °C) of Fraction V products

	5% Albumin		25% Albumin		HPPS	
Time at 60 °C (h)	WNV[a]	BVDV[b]	WNV[a]	BVDV[b]	WNV[a]	VV[a]
Unheated[a]	7·4	4·8	6·7	4·4	7·4	6·0
0·5	0·8	≤ 0·7	≤ 0·3	≤ 0·7	≤ 0·3	1·7
1	0·2	≤ 0·7	≤ 0·3	≤ 0·7	≤ 0·3	0·8
2	≤ 0·3	≤ 0·7	≤ 0·3	≤ 0·7	≤ 0·3	≤ 0·3
5	≤ 0·3	≤ 0·7	≤ 0·3	≤ 0·7	≤ 0·3	≤ 0·3
10	≤ 0·0[d]	≤ 0·7	≤ 0·0[d]	≤ 0·7	≤ 0·3	≤ 0·3
$\log_{10}$ reduction factor	≥ 7·4	≥ 4·1	≥ 6·7	≥ 3·7	≥ 7·1	≥ 5·7

[a]Data from the West Nile virus (WNV) and vaccinia virus (VV) samples are expressed as $\log_{10}$ plaque-forming units/ml.
[b]Data from the bovine viral diarrhoea virus (BVDV) samples are expressed as $\log_{10}$ tissue culture infectious dose 50/ml.
[c]Virus was spiked into samples once they reached 60 °C. As the potential existed for immediate inactivation, the initial virus concentration was determined from an unheated sample.
[d]Increased volume testing.
WNV values represent the mean of three determinations, BVDV values represent the mean of two (albumin) or four [human plasma protein solution (HPPS)] determinations and VV values represent a single determination.

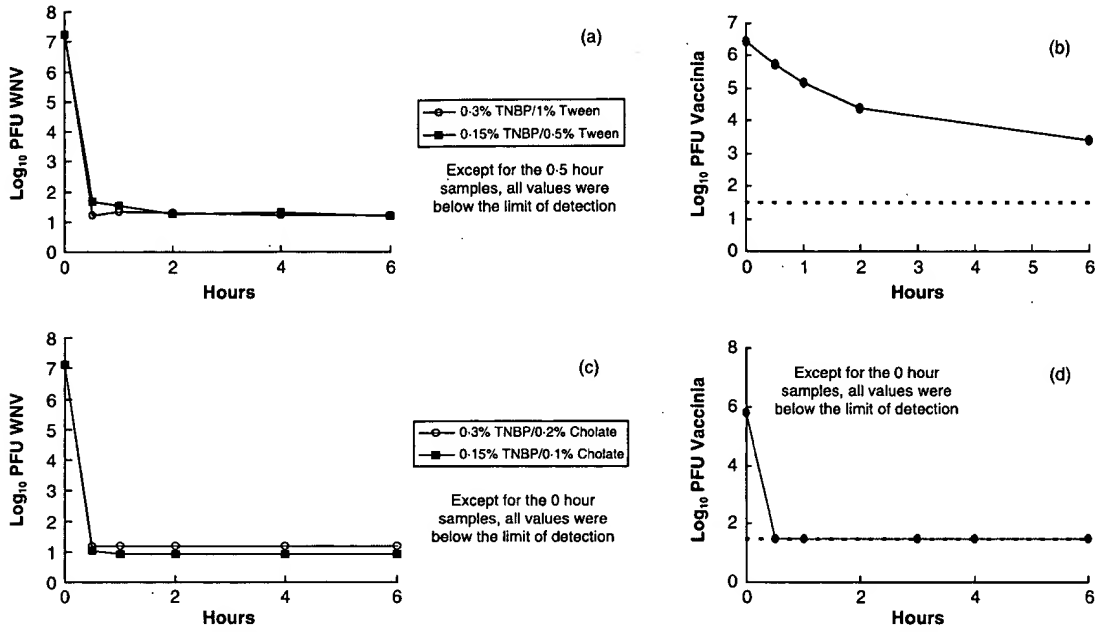

Fig. 1 Inactivation of West Nile virus (WNV) and vaccinia virus (VV) in solvent/detergent. Solutions of anti-haemophilic factor (AHF) were treated with tri-(*n*-butyl)-phosphate (TNBP)/Tween 80 and the inactivation of infectious (a) WNV in 0·3% TNBP/1% Tween 80 or 0·15% TNBP/0·5% Tween 80 or (b) VV in 0·3% TNBP/1% Tween 80 was determined. For solutions of intravenous immunoglobulin produced using the solvent/detergent process (IVIG-S/D), the inactivation of (c) WNV in 0·3% TNBP/0·2% cholate or 0·15% TNBP/0·1% cholate or (d) VV in 0·3% TNBP/0·2% cholate was determined. Each WNV point represents the mean of three determinations and each VV point represents a single determination. The dashed lines in panels (b) and (d) represent the limit of detection. Overall reduction factors are presented in Table 3.

When 5% or 25% albumin was spiked with either WNV or BVDV and pasteurized at 60 °C for 10 h, all infectious virus was inactivated (Table 2). Within 30 min of incubation of 5% albumin at 60 °C, WNV was near the lower limit of detection and no BVDV could be detected. Pasteurization of 5% albumin inactivated ≥ 7·4 $\log_{10}$ WNV and ≥ 4·1 $\log_{10}$ BVDV. Similarly, pasteurization of 25% albumin resulted in the complete inactivation of both WNV and BVDV within 30 min. A total of ≥ 6·7 $\log_{10}$ WNV and ≥ 3·7 $\log_{10}$ BVDV were cleared.

Solvent/detergent treatment

Within 1 h of incubation of the AHF intermediate with either 0·3% TNBP/1% Tween 80 or 0·15% TNBP/0·5% Tween 80, no infectious WNV could be detected (Fig. 1a). The production operating concentration of 0·3% TNBP/1% Tween 80 resulted in ≥ 5·9 $\log_{10}$ inactivation of WNV, and when the concentration was diluted twofold, ≥ 5·9 $\log_{10}$ inactivation was also observed (Table 3). Solutions of AHF spiked with BVDV and incubated in 0·3% TNBP/1% Tween 80 or 0·15% TNBP/0·5% Tween 80 resulted in ≥ 5·3 $\log_{10}$ and ≥ 5·2 $\log_{10}$ inactivation, respectively (Table 3). In contrast, VV was more resistant to inactivation under these conditions. Following 6 h of incubation in 0·3% TNBP/1% Tween 80, 3·0 $\log_{10}$ inactivation was achieved (Fig. 1b, Table 3).

Table 3 Virus inactivation by tri-(*n*-butyl)-phosphate (TNBP)/Tween 80 in solutions of anti-haemophilic factor (AHF) or TNBP/cholate in intravenous immunoglobulin produced using the solvent/detergent process (IVIG-S/D)

		$\log_{10}$ inactivation		
Protein	**Inactivation step**	**WNV**	**BVDV**	**VV**
AHF	0·3% TNBP/1% Tween 80	≥ 5·9[a]	≥ 5·3	3·0[b]
	0·15% TNBP/0·5% Tween 80	≥ 5·9[a]	≥ 5·2	ND[c]
IVIG-S/D	0·3% TNBP/0·2% Cholate	≥ 5·9[d]	≥ 4·2	≥ 4·6[e]
	0·15% TNBP/0·1% Cholate	≥ 6·2[d]	≥ 3·9	ND

[a]From Fig. 1(a).
[b]From Fig. 1(b).
[c]ND = not determined.
[d]From Fig. 1(c).
[e]From Fig. 1(d).
For West Nile virus (WNV), each value represents the mean of three determinations; for bovine viral diarrhoea virus (BVDV), each value represents the mean of two or three determinations; and for vaccinia virus (VV), the value is the result of a single determination.
Solvent/detergent solutions were incubated at 28 °C for 6 h.

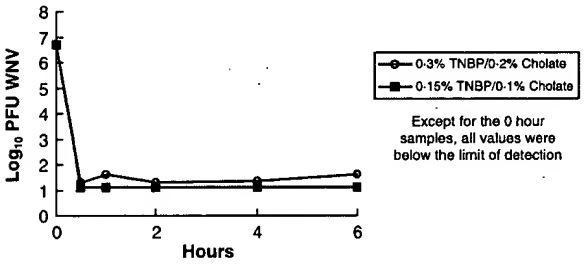

Fig. 2 Kinetics of West Nile virus (WNV) inactivation during the treatment of intramuscular immunoglobulin (IMIG) with 0·3% tri-(*n*-butyl)-phosphate (TNBP)/0·2% cholate or 0·15% TNBP/0·1% cholate. Each point represents the mean of three determinations. Total inactivations of ≥ 5·4 $\log_{10}$ and ≥ 5·6 $\log_{10}$ WNV were achieved in 0·3% TNBP/0·2% cholate and 0·15% TNBP/0·1% cholate, respectively.

Incubation of WNV in an IVIG-S/D process intermediate solution, containing either 0·3% TNBP/0·2% cholate (manufacturing conditions) or 0·15% TNBP/0·1% cholate, resulted in complete inactivation of the virus within 30 min (Fig. 1c). Approximately 6 $\log_{10}$ of WNV was inactivated (Table 3). Similarly, in 0·3% TNBP/0·2% cholate or 0·15% TNBP/0·1% cholate, ≥ 4·2 $\log_{10}$ and ≥ 3·9 $\log_{10}$ BVDV inactivation, respectively, was observed (Table 3). Unlike the observations with TNBP/Tween 80, complete and rapid inactivation of VV was achieved during the treatment of IVIG-S/D with 0·3% TNBP/0·2% cholate. Within 30 min, > 4 $\log_{10}$ of inactivation was achieved and, by 3 h, no infectious virus could be detected (Fig. 1d). A total of ≥ 4·6 $\log_{10}$ of VV was inactivated (Table 3).

Similarly, WNV was readily inactivated when the IGIM process intermediate was incubated in either 0·3% TNBP/0·2% cholate (manufacturing conditions) or 0·15% TNBP/0·1% cholate (Fig. 2). Complete inactivation was observed within 30 min of incubation. Total inactivation of ≥ 5·4 $\log_{10}$ and ≥ 5·6 $\log_{10}$ were achieved in 0·3% TNBP/0·2% cholate and 0·15% TNBP/0·1% cholate, respectively.

Caprylate treatment

Treatment of a Gamunex® intermediate solution with sodium caprylate resulted in the complete inactivation of both WNV and VV within 3 min. In 14 mM caprylate at 22 °C, ≥ 5·0 $\log_{10}$ WNV was inactivated (Fig. 3a, Table 4). When incubated at 22 °C in 20 mM caprylate, ≥ 6·0 $\log_{10}$ VV was inactivated (Fig. 3b, Table 4).

Caprylate has previously been shown to provide robust and effective inactivation of human immunodeficiency virus (HIV), pseudorabies virus (PRV) and BVDV [10]. To further evaluate the efficacy of this innovative method for the inactivation of enveloped virus, additional models for HBV and HCV were used in inactivation studies (Table 4). The data demonstrated that no infectious DHBV, an HBV surrogate, could be detected following incubation in 20 mM caprylate. Within 3 min, DHBV inactivation was complete (kinetics not shown). Another model

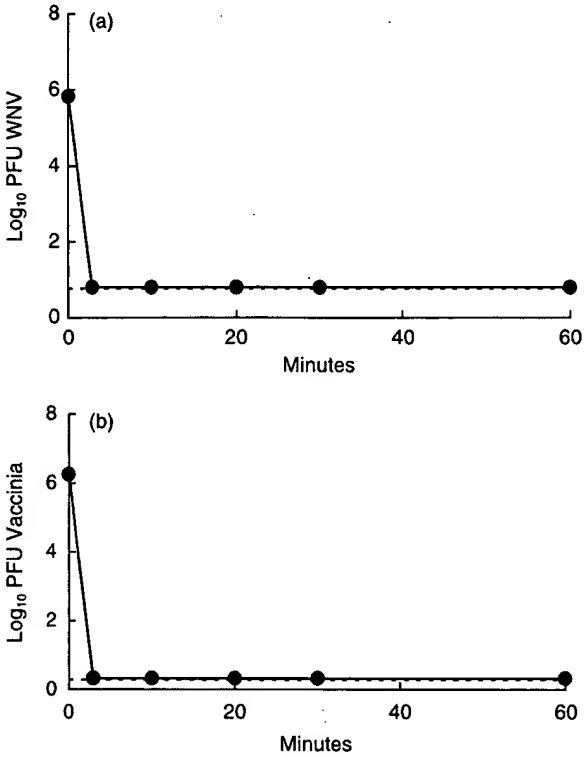

Fig. 3 Kinetics of West Nile virus (WNV) (a) and vaccinia virus (VV) (b) inactivation during the incubation of Gamunex® in 20 mM caprylate. Except for the 0-h samples, all values were below the limit of detection. The dashed line represents the lower limit of assay detection. For WNV, each point represents the mean of three determinations and, for VV, each point is a single determination. Overall reduction factors are presented in Table 4.

for HCV – Sindbis – was similarly shown to be rapidly and completely inactivated in 18 mM caprylate.

Inactivation of HIV, PRV and BVDV have previously been shown to be robust with respect to caprylate concentration [10]. Robust inactivation of BVDV with respect to protein concentration, pH and incubation temperature have also been demonstrated [10]. To further investigate the robustness of caprylate inactivation of enveloped viruses, a series of studies were performed to evaluate the robustness of Sindbis virus inactivation with respect to caprylate concentration, protein concentration, pH and incubation temperature. These data, shown in Table 5, demonstrate that within the ranges investigated, these operating parameters did not influence Sindbis inactivation by caprylate. Under all conditions, complete inactivation of Sindbis was observed within 10 min (kinetics not shown).

Low-pH incubation

Incubation of the Gamunex® final product (pH 4·4) at 23 °C, resulted in the effective inactivation of WNV (Fig. 4). Within

Table 4 Inactivation of enveloped viruses in the Gamunex® process intermediate containing 20 mM caprylate during incubation at 24 °C: within 10 min, all viruses were undetectable

Virus	Models	Log_{10} reduction
HIV[a]	HIV-1, HIV-2	≥ 4·5
PRV[a]	Human herpes viruses, HBV	≥ 4·6
BVDV[a]	HCV	≥ 4·5
Sindbis[b]	HCV	≥ 6·0
DHBV	HBV	≥ 4·6
WNV[c]	Relevant	≥ 5·0
VV[d]	Relevant, variola, monkeypox	≥ 6·0

[a]Data obtained from a previously published reference [18].
[b]From Table 5.
[c]From Fig. 3(a).
[d]From Fig. 3(b).
BVDV, bovine viral diarrhoea virus; DHBV, duck hepatitis B virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; PRV, pseudorabies virus; WNV, West Nile virus; VV, vaccinia virus.
The reduction factor for VV represents a single determination. All other reduction factors were the mean of three determinations.

Table 5 Robustness of Sindbis virus inactivation with respect to caprylate concentration, protein concentration, pH and incubation temperature during the caprylate-inactivation step of the Gamunex® process

Parameter	Parameter level	Log_{10} Sindbis inactivation
Caprylate concentration	18 mM	≥ 6·0
	15 mM	≥ 5·9
	11 mM	≥ 5·9
Protein concentration	1·1%	≥ 5·8
	1·2%	**≥ 6·0**
	1·5%	≥ 5·8
pH	4·9	≥ 6·3
	5·1	**≥ 6·0**
	5·3	≥ 6·3
Temperature	**25 °C**	**≥ 6·0**
	20 °C	≥ 5·8

One parameter was varied at a time, while all others were held at the production-operating setpoint (shown in bold; 20 mM caprylate, pH 5·10, 1·2% protein, 25 °C). Each value represents the mean of three determinations.

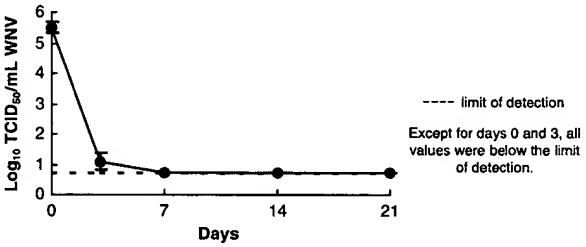

Fig. 4 Kinetics of West Nile virus (WNV) inactivation during low-pH incubation of Gamunex®. WNV was spiked into the Gamunex® final product that had been adjusted to pH 4·4, and was incubated for 21 days at 23 °C. Virus infectivity was monitored by removing samples for titration at 0, 3, 7, 14 and 21 days after incubation began. The value at each time point represents the mean + 1 SD of three determinations. A total of ≥ 4·8 log_{10} WNV inactivation was achieved.

3 days of incubation at pH 4·4, WNV was near the lower limit of detection and, by the next time-point (7 days), complete inactivation was achieved. A total of ≥ 4·8 log_{10} inactivation was observed.

Discussion

Effective inactivation of WNV, BVDV and VV by pasteurization was demonstrated in a variety of protein solutions, even in the presence of a stabilizer. Although albumin, HPPS and α_1-PI utilize different excipients for stabilization (e.g. sucrose, citrate, tryptophan) and were at different protein concentrations, complete inactivation of the viruses was achieved, demonstrating that this technology provides robust inactivation of enveloped viruses. As the complete inactivation of WNV, BVDV and VV was observed in both 5% and 25% albumin, similar inactivation of these viruses would be expected for all intermediate concentrations.

Both WNV and VV have previously been shown to be susceptible to inactivation by heat. Complete inactivation of VV was observed during the pasteurization of solutions of immunoglobulin at 60 °C [14,15], and a recent report has demonstrated the inactivation of WNV during the pasteurization of albumin [16]. A number of studies, including this one, have demonstrated the inactivation, by pasteurization, of viruses that are closely related to WNV, such as BVDV, tick borne encephalitis virus, yellow fever virus and Sindbis virus [16,17,19]. The data reported here, which demonstrated a

similar inactivation of WNV and BVDV by pasteurization of α_1-PI and albumin, support the predictive value of inactivation information from model viruses. VV is antigenically similar enough to both variola (smallpox) and monkeypox viruses to be used as a vaccine [7]. The susceptibilities to pasteurization of monkeypox virus and variola virus, then, are probably similar to that of VV.

Solvent/detergent treatment is widely used to inactivate lipid-enveloped viruses. Treatment of the AHF intermediate with 50% of the TNBP/Tween 80 concentration used in the manufacturing process provided rapid and complete inactivation of WNV. Again, these results were very similar to those of the related virus, BVDV, supporting the validity of this virus as a model for WNV.

VV, however, was more resistant to inactivation by TNBP/Tween 80. The production-operating concentration of TNBP/Tween 80 did not achieve complete inactivation of VV, although the virus titre was reduced by over 3 $\log_{10}$. The resistance of VV to solvent/detergent has previously been observed [18,19]. VV is unlike other enveloped viruses in that it can be present in two infectious forms, both of which are membranous and one of which is a double membrane with a complex structure [9,20].

In contrast to TNBP/Tween 80, in this study, TNBP/cholate provided effective inactivation of both WNV and VV. Treatment of human placental tissue with 0·3% TNBP/0·2% cholate at 4 °C resulted in substantial, but incomplete, VV inactivation [21]. In the current study, TNBP/cholate in a solution of IVIG, rather than tissue, was incubated at 28 °C, and complete inactivation of the virus was achieved. Cholate may be able to solubilize VV membranes more efficiently than other detergents. Thus, these TNBP/cholate data provide evidence of the ability of this method to effectively inactivate enveloped viruses with a wide variety of characteristics. It also suggests that an emergent enveloped virus with characteristics similar to the viruses for which TNBP/cholate data exist will also be susceptible to inactivation by this method.

WNV was effectively inactivated during the low-pH incubation step of the Gamunex® process, even when the incubation was performed at pH 4·4, above the upper pH limit of the manufacturing range (pH 4·0–4·3). The susceptibility of this virus to low-pH-mediated inactivation has previously been demonstrated, and the current data confirm that incubation of this product under conditions of low pH similarly achieve inactivation of the virus [16,22]. It is likely that any manufacturing step that was performed at a low pH would effectively inactivate WNV. Vaccinia has also been shown to be susceptible to inactivation at low pH [23].

Caprylate treatment provided rapid and complete inactivation of the wide panel of enveloped viruses that were evaluated. To date, all enveloped viruses evaluated, representing a wide variety of physicochemical characteristics, have been inactivated to the limit of detection within minutes of incubation in caprylate. Caprylate inactivation of VV was similar to that of other enveloped viruses, demonstrating that it can be an alternative to solvent/detergent treatment for the inactivation of resistant enveloped viruses. These data also suggest that any emergent enveloped virus with a complex lipid membrane may be similarly susceptible to this means of inactivation.

The data presented here support the robust inactivation of enveloped viruses by caprylate. A number of enveloped viruses, representing a variety of virus families, were shown to be completely inactivated within minutes of incubation with caprylate. In addition, data obtained using Sindbis virus demonstrated that during the Gamunex® manufacturing process, variations in caprylate concentration, protein concentration, pH or incubation temperature would not alter the overall inactivation or the inactivation kinetics of this HCV surrogate.

These data also demonstrated the validity of the use of model viruses for inactivation studies. Inactivation of WNV by pasteurization, solvent/detergent treatment and caprylate treatment was very similar to that observed with BVDV in this study. The use of surrogate viruses for those viruses that cannot readily cultured *in vitro* is a widely used strategy that has been advocated by regulatory agencies [1]. BVDV has been used as a model for HCV for many years, and its ability to predict the inactivation of HCV has been verified by the lack of transmission of this pathogen by blood products subjected to inactivation measures that achieve significant levels of BVDV inactivation. The WNV data presented here support the use of model viruses; WNV inactivation mirrored that of BVDV. The appearance of a novel viral pathogen should not place the safety of plasma-derived products in jeopardy as long as data from viruses with similar physicochemical characteristics support effective inactivation during manufacture. Data from studies with a wide panel of model viruses, representing a range of physicochemical properties, should provide a basis for determining whether an emergent pathogen is likely to present a threat to biological products.

Although data from model viruses was predictive of the safety of plasma-derived products with respect to transmission of WNV, VV or other potential viral contaminants with similar properties, the results of the current study provide definitive confirmation. This is consistent with the conclusion of others [16]. Furthermore, these data justify the model virus approach for assessing the viral safety of these products.

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Pathogen inactivation of platelet concentrates and fresh frozen plasma

J E JACKSON
Department of Transfusion Medicine, Leicester Royal Infirmary, Infirmary Square, Leicester LE1 5WW, UK

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ABSTRACT

Transfusion of blood products carries the risk of pathogen transmission, despite careful donor selection and screening tests. This is due to viral transmission from window period donations, the emergence of new pathogens such as variant Creutzfeldt-Jakob disease, for which routine screening tests are not yet available, and to bacterial contamination. Techniques have been developed to inactivate pathogens in both fresh frozen plasma and platelet concentrates. The relative benefits to the recipient, and the ease of incorporation into blood component processing are considered for the technologies currently available.

KEY WORDS: Blood-borne pathogens.
Plasma.
Platelet transfusion.

Introduction

The risk of viral transmission by transfusion has been reduced with the introduction of careful selection procedures for blood donors, and with the implementation of screening tests for known bloodborne pathogens for each blood donation.[1]

However, although these strategies have significantly increased the safety of the blood supply in developed countries, there remains a residual risk of viral transmission. This is due to the so-called window period or lag phase between donor infection and the point at which seroconversion gives rise to a positive screening result. Nucleic acid technology has reduced this window period to between 8–11 days for human immunodeficiency virus (HIV) and hepatitis C infection.[2]

The second concern is the emergence of new infectious agents such as variant Creutzfeldt-Jakob disease (vCJD), the bloodborne hepatitis virus that is transfusion-transmitted (TTV) with unknown clinical significance[3] and West Nile virus, an outbreak of which occurred in New York.[4]

While the safety of the blood supply has improved and the risk of viral transmission has diminished, the opposite is true for bacterial contamination. This was the first recognised infectious hazard of transfusion and remains an ongoing problem. Sepsis and mortality can result from the transfusion of contaminated blood products. Platelet concentrates are especially implicated due to their storage at room temperature.[5] It has been estimated that the risk of acquiring an infectious disease following a transfusion of five blood components is approximately 2.7/1000 patients transfused.[6]

Products derived from fractionated plasma undergo viral inactivation steps as part of the manufacturing process, but these methods are inappropriate for more labile blood products such as platelet concentrates and fresh frozen plasma (FFP).[7] Recently, techniques have been developed to inactivate pathogens in both platelet concentrates and FFP[8,9] and it is considered that the introduction of these into the manufacturing process would ensure the safety of these blood products to an even greater extent.

Correspondence to: Mrs Jane Jackson
Email: jane.jackson@rohl-tr.nhs.uk
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Infectious pathogens in blood

Blood can harbour many pathogens, including enveloped and non-enveloped viruses, bacteria, parasites and possibly prions.[7] Viruses can exist in blood as cell-free forms in plasma, cell-associated forms (either in or on leucocytes), or integrated into the genomic nucleic acids of cells such as leucocytes and megakaryocytes as a latent proviral form.[4]

Blood donations are currently screened for hepatitis B surface antigen, anti-HIV-1 and -2, anti-hepatitis C virus, and hepatitis C nucleic acid. A proportion of donations are also screened for anti-cytomegalovirus (CMV).

The incidence of viral transmission by transfusion is now so rare, however, that retrospective studies no longer can be used to assess risk levels. Mathematical models can be employed to give an indication of the level of risk. Estimates for the risk of viral transmission per unit, based on mathematical models, are 1/1.8 million units for HIV, 1/1.6 million for hepatitis C virus (HCV) and 1/220 000 for hepatitis B virus (HBV).[2]

There are also known bloodborne viruses that are not screened for, such as parvovirus B19, hepatitis G and various herpes viruses. While their clinical significance is unclear in the general population, there are certain categories of patient in whom transmission of these viruses is undesirable.[1]

Blood is also screened for antibodies to *Treponema pallidum*, the causative agent of syphilis. This is not thought to be of significant risk for transfusion purposes, but the continued inclusion of this test might serve to identify blood donors with undesirable lifestyles.[1]

Post-transfusion bacterial infection usually results from bacterial contamination, often due to the introduction of

bacteria from the donor's skin.[5] In several countries bacterial contamination is the most frequently encountered transfusion complication; however, the level is difficult to establish due to under reporting.[7]

Yomtovian has likened the problem to an iceberg,[8] because only the tip, representing the clinically significant cases, is apparent. In reality, the level of contamination is much higher, but not all cases produce symptoms, and not all symptoms can be attributed to a transfusion. Thus, the actual frequency of bacterial contamination is hard to assess, but is estimated to be approximately 1/2400 single donor platelet units.[9]

There are commercially available products to screen for bacterial contamination and their use is to be mandated in the USA. Bacterial contamination can be detected, but testing requires sampling at time intervals up to 48 hours, therefore a holding phase is required.[9] Blood collection bags that divert the first 20 mL of the donation minimise the introduction of skin bacteria.[10]

Creutzfeldt-Jakob disease and vCJD, so-called mad cow disease, are human forms of spongiform encephalopathy caused by infectious prion proteins.[1] Currently, no screening technique has been introduced into routine use, but possible testing systems are under development.[7]

Possible prion transmission route is thought to be via the B lymphocytes, a route suggested by limited experimental data that show that mice lacking mature B lymphocytes do not become infected with prions.[1] This data is the major factor in the decision to leucodeplete all donated blood in the UK and Ireland.[11]

Perhaps surprisingly, contaminating leucocytes are also considered as pathogens, and as such are targets for inactivation. This is because they can be the source of infection and give rise to an untoward transfusion outcome.[7] Their presence is undesirable for several reasons. The first is that leucocytes may harbour and transmit cell-bound infectious agents such as CMV and HIV, and may be involved in the transmission of prions. While this is a major consideration for pathogen inactivation, transfusion of contaminating leucocytes can also cause other side-effects such as febrile non-haemolytic transfusion reactions (FNHTR), graft versus host disease (GVHD), and, as a consequence of immune modulation, post-operative infections and tumour reoccurrence.

The introduction of leucodepletion as part of the blood processing scheme is designed to reduce the risk of immune and viral complications, but is not adequate to prevent transfusion-associated GVHD (TA-GVHD) or CMV infection.[6]

Process requirements

An effective pathogen inactivation technique would achieve greater transfusion safety than any further refinements in microbiology testing because more pathogens can be inactivated than are currently tested for. The advantage of this is that as new pathogens enter the donor supply, its safety would be maintained by the further development of inactivation procedures.[6] However, the addition of any substance to blood products is not without risk, and several factors must be considered before a particular technique for pathogen inactivation is used routinely.

Areas that need to be studied carefully include the efficacy of the process, as ideally all targets need to be inactivated, and whether the blood product is damaged or altered by the process, as FFP and platelet concentrates need to be functional when transfused. The process used must be non-toxic to the recipient. The safety and efficacy need to be assessed by *in vitro* and *in vivo* tests, and successful clinical trials have to be undertaken.

It is expected that there should be some cost to health benefit in undertaking the procedure, as the introduction of expensive extra steps in the manufacturing process should provide additional safety and health benefits to the recipient.

Procedures currently at various stages of development can be loosely divided into two categories: photoinactivation and what are broadly classed as 'new technologies'. Photoinactivation methods are based on the use of photosensitisers, which are dyes that have light absorption properties if illuminated by a specific light source. This can lead to photodynamic reactions in which active oxygen species disrupt the viral envelope, or to photochemical reactions in which the pathogenic nucleic acid is altered irreversibly. So-called new technologies are based on compounds that modify cellular or viral nucleic acids irreversibly, but do not necessarily need an external energy source.[7]

Platelet concentrates

Recognition of potential bacterial contamination of platelet concentrates due to the rapid replication of bacteria at room temperature led to the reduction in *in vitro* storage of platelets from 7 to 5 days.[6]

In addition to targeting the known viral risks, any system for pathogen inactivation used on platelets needs to act against a broad spectrum of bacteria to eliminate them and inhibit any regrowth during the shelf-life of the product. Platelet function needs to be intact, and there must be an adequate increase in the platelet count post-transfusion.[6]

In vitro platelet assays are commonly used, but there is some doubt as to whether or not they predict platelet recovery and survival post-transfusion. The most sensitive and specific assessment is thought to include morphology score, shape changes, hypotonic shock reversal, (which correlates well with recovery, but not lifespan) and adenosine triphosphate content. pH is also important because if it drops below 6.2 then platelet life span is reduced. Although assessment using these criteria is important, satisfactory results do not guarantee haemostasis, the clinical evaluation of which is difficult to assess.[6]

Gamma irradiation (25 Gray) to inactivate lymphocytes is used to prevent TA-GVHD following platelet transfusion.[12] This dose is insufficient to inactivate any microbial pathogens in the platelet concentrate. The dose that would be needed to achieve this would make platelets non-viable, and therefore this technique cannot be considered for pathogen inactivation.[7]

Photochemical treatment

Several potential inactivation techniques applicable to platelet concentrates have been investigated. Merocyanine 540, which targets viral envelopes, leads to platelet

activation and serotonin release, so its use was not pursued further. Thionine also has pathogen inactivation properties when excited by light at 590 nm, and its use is being explored by German scientists.[6]

The main focus of interest has been on a group of compounds known as psoralens. These are planar furocoumarins, many of which are synthesised by plants, are present in vegetables such as celery, and have little known toxicity.

Psoralens bind reversibly with both single- and double-strand nucleic acids by intercalation, and then react with UVA light to inactivate pathogens by an irreversible photochemical reaction in which monoadducts and crosslinks are formed.[15] Different psoralen structures lead to differences in nucleic acid binding constants and therefore different pathogen inactivation efficiency.

Binding inhibits nucleic acid replication, transcription and translation. This is not specific to pathogenic DNA but is beneficial,[7] as nucleic cell function is not vital for transfused platelets and nucleic acid disruption in any residual leucocytes will diminish the occurrence of FNHTR, TA-GVHD and adverse immune responses.

The first psoralen studied was methoxypsoralen (8-MOP) which targets all three viral forms, as well as Gram-positive and Gram-negative bacteria and protozoa. As a result of the photochemical reactions with UVA light, adduct formation with nucleic acid is observed, but binding is of low affinity and competitive binding with plasma proteins is seen. Long illumination times, reduced oxygen levels and suspension of platelets in a non-protein medium are needed to achieve pathogen inactivation. This is not satisfactory for routine use so this compound has not been developed further.[6]

Another psoralen studied is a synthetic compound known as aminomethyl trimethyl psoralen (AMT). This is effective for viral inactivation, but its action against bacteria is unknown. However, considerable UVA illumination is needed and free radical quenchers such as rutin must be added to prevent the active oxygen species formed damaging the platelets. This adds to the complexity of the system. Toxicology studies also reveal that residual AMT (the remnants following photochemical treatment) has mutagenic potential in the absence of light.[6]

A number of psoralens have been synthesised and from these an aminoalkylated psoralen, originally termed S-59 but now known as amotosalen,[16] was chosen because of its favourable toxicology profile. Structurally, it combines the characteristics of 8-MOP and AMT.[6]

Infectious pathogens are rapidly inactivated due to the high binding affinity of amotosalen with nucleic acids. Using a platelet additive solution to reduce the plasma concentration, platelet function is maintained. Also, there is no need for the addition of quenchers.

The pathogen inactivation potential of amotosalen and UVA light was determined by adding high levels of pathogens to platelet concentrates, and using bioassays to assess the level of infectivity after treatment.[15] HIV-1, hepatitis B and C, and examples of both Gram-positive and Gram-negative bacteria are all inactivated following illumination by 3J/cm² UVA light.[15]

In vitro platelet function was assessed by comparing treated and non-treated platelet concentrates. Treated platelets showed similar results to non-treated platelets, apart from a difference in P-selectin expression, which may be a predictor of shortened survival post-transfusion.[6]

Clinical trials have been completed successfully, firstly using autologous radiolabelled platelets in healthy volunteers. Although the results showed decreased platelet recovery and lifespan, they were tolerated without adverse incident. Treated platelets have also undergone trials in patients with thrombocytopenia, which proves that haemostatic function is maintained.[7] Extensive evaluations have been undertaken both in Europe and in the USA with no problems observed in haemostatic ability, recovery or survival.[15]

There has been some concern about whether or not photochemical treatment of platelets will lead to the production of neoantigens, which could cause problems for patients receiving multiple platelet transfusions, but evidence produced so far does not indicated this.[6]

The INTERCEPT system, which employs amotosalen, has been developed by Cerus in collaboration with Baxter Healthcare.[16] It inactivates pathogens in platelet concentrates by what is known as Helinx technology, and is the only system ready for the commercial market. A phase III trial (the euroSPRITE trial) has been completed using pooled buffy coat platelets, and no differences were observed in haemostatic function or adverse events between treated and non-treated platelets.[17]

The system has obtained a CE mark and process evaluation for European blood bank good manufacturing practice requirements has been undertaken.[18] Currently, introduction of this technology is awaiting government approval. An additional benefit of the INTERCEPT system is that gamma irradiation is not required, as the inactivation technique prevents GVHD.

In the USA, platelets are prepared by apheresis or from platelet-rich plasma derived from whole blood; therefore, due to this variance in production, further clinical trials are required before it can be considered for this market. It is possible that pathogen inactivation may be used in addition to detection tests.[19]

Fresh frozen plasma

Fresh frozen plasma is used to treat congenital coagulation deficiencies when no specific coagulation factor is available, and also for acquired coagulation deficiencies because it contains all the coagulation factors and inhibitors normally present in plasma. First-time donations are not used to prepare FFP in order to minimise viral risk.[11]

Viruses are the main target for pathogen inactivation in FFP, especially those that can exist in a cell-free form in plasma. Any inactivation technique employed needs to maintain the functionality of the product at an adequate level. This can easily be assessed *in vitro* by clotting screens that measure coagulation pathways (e.g., prothrombin time), by coagulation factor assays that measure the level of specific factors, and by assays that test for the presence of inhibitors of haemostasis (e.g., antithrombin III, and proteins C and S).

Tests can also be performed to detect markers which signify activation of the product.[7] *In vivo* tests used to ensure the efficacy of the product include pharmacokinetic studies in volunteers who have received an FFP transfusion, and also by assessing the response when the product is

transfused into patients with known coagulopathies.[7]

Viral inactivation of FFP is more advanced than for platelet concentrates, as there are currently two virally inactivated products available in the UK. The two processes used are methylene blue treatment and solvent detergent treatment.[11]

Methylene blue treatment

Methylene blue, in combination with visible light, has pathogen inactivating properties. It is a hydrophilic dye that undergoes a photodynamic reaction with light to form a reactive oxygen species. This leads to oxygen depletion and cell damage. Viruses are killed by the energy transfer reactions involving oxygen in this excited state.[7]

The process inactivates enveloped viruses by damaging their nucleic acids, preventing replication; however, it is unclear whether or not non-enveloped viruses are inactivated.[11] There is some evidence to suggest that methylene blue is effective against parvovirus B19,[7] and it is estimated that 50% of prion proteins are removed by the filtration step that is part of treatment.[19]

Methylene blue treatment affects several labile plasma products such as FVIII, but the levels obtained, although reduced by 15–20%, remain within acceptable limits;[8] however, concern has been raised that only plasma from group A donors, who are known to have higher levels of FVIII, would maintain acceptable levels after treatment.[19] Fibrinogen is also susceptible to photo-oxidative damage, and some reports have suggested up to 39% reduction in activity.[20]

As methylene blue is not effective against cell-associated viruses, any residual cells are first removed by filtration, and then the dye is added and the pack illuminated. Techniques are now being developed so that both sides of the pack can be illuminated at the same time, and up to three packs processed together to speed up the procedure.[19]

There are concerns about the toxicity of methylene blue, but it has been used therapeutically at much higher levels, with no resulting toxicity observed, and there are processes available to remove the methylene blue if required. Over two million methylene blue-treated plasma donations have been transfused across Europe, without adverse reaction;[11] however, it is no longer accepted by European regulatory authorities, due to concerns about mutagenicity, and is currently only used in the UK.[14,20]

Owing to the unknown risk of vCJD transmission, methylene blue-treated plasma for use in neonates and children born after 1 January 1996 has been available in the UK since July 2002.[21] This product was chosen as it is the only single-unit, pathogen-inactivated plasma available.

In order to limit donor exposure further and allow additional product criteria to be developed, the National Blood Service (NBS) uses a panel of accredited donors for the preparation of blood components for paediatric and neonatal use.[21]

Soon, the plasma used will be sourced in North America, following Department of Health recommendation. If this is frozen prior to import and methylene blue treatment in the UK, a further loss in coagulation factor activity is expected.[22]

Owing to a lack of data on clinical efficacy and tolerance, the equivalency of methylene blue-treated plasma and FFP has not been demonstrated satisfactorily.[24]

Solvent detergent treatment

The other virally inactivated product available for use in the UK is solvent detergent-treated FFP. This is used widely in Europe, and in countries such as Norway and Belgium its use has completely replaced that of FFP.[8] In addition, the Food and Drug Administration has recommended approval of this product in the USA.[8]

Lipid-enveloped viruses can be differentiated from protein-coated viruses by the use of solvent ethyl ether and the detergent Tween 80. This forms the basis on which viral inactivation using solvents and detergents has been developed. Different solvent and detergent combinations have been evaluated for their ability to inactivate enveloped viruses without detriment to FFP function.

The method currently in use involves treatment with 1% Tri (N-butyl) phosphate (TNBP) and 1% Triton x-100.[8,25] These are removed using vegetable oil extraction and reverse phase chromatography with a C18 resin during the purification process, and the levels remaining are not expected to have any clinical side effects.

Solvent detergent treatment is effective against the majority of transfusion-transmitted viruses (e.g., HIV, HBV and HCV) as these are enveloped viruses. Solvent detergent treatment is not effective against non-enveloped viruses, which means that hepatitis A (HAV) virus and parvovirus B19 may not be inactivated by this process. It is also suspected that prion proteins are not inactivated by this treatment.[26]

Solvent detergent treatment is used to process large pools of ABO-identical plasma donations, and some 600–1500 donations are pooled in Europe and up to 2500 in the USA.[11]

Presence of HAV and parvovirus B19 will be diluted in the process and it may provide partial protection due to the presence of antibodies to these viruses in the plasma pool.[27] There have been clinical cases of parvovirus seroconversion reported following the transfusion of this product.[7]

Pooling the plasma may reduce the risk of post-transfusion complications such as allergic reactions and transfusion-related acute lung injury (TRALI).[11]

As seen with the use of methylene blue, solvent detergent treatment results in a decrease in coagulation factor activity.[27] FVIII activity is reduced by over 20% and levels of protein S and α-2 antiplasmin fall by more than 50%. The process also removes high-molecular-weight von Willebrand factor multimers, which makes it particularly suitable for the treatment of thrombotic thrombocytopenic purpura (TTP).[27] As the batch product is treated in its entirety, the levels of coagulation factors are consistent throughout.[11]

The major concern about the use of solvent detergent-treated FFP seems to be that it is a pooled product. Although this may convey some benefits, the risk of parvovirus B19 transmission may mean that it is not the product of choice for certain at-risk categories of patient, such as pregnant women and patients with severe immunodeficiencies and haemolytic anaemia.[8]

Solvent detergent-treatment only inactivates enveloped viruses and although the process reduces the viral risk from currently known pathogens, there is always the risk that newly emerging ones may not be inactivated. The use of a pooled product then provides the potential for widespread transmission.[8]

It has been suggested that in order to satisfy all categories of patient it may be necessary to stock a variety of FFP in the blood bank.[9]

Pharmacoeconomics

While pathogen inactivation is considered to be the way forward, the pursuit of zero risk must be balanced against cost-effectiveness.

Pharmacoeconomic studies have considered several factors. The selection of donors and the screening of donations already includes a high safety margin, thus any further improvements will be slight, hard to prove and expensive, resulting in a marginal cost:benefit ratio.

Clinical trials do not necessarily show equivalency with non-treated products. Greater volumes of raw materials (up to 30%) may have to be used in the manufacturing process to achieve an 'equivalent' product. This has obvious economic implications, and supply may be a problem with an ever-decreasing donor population.[23]

Cost effectiveness studies in this area are limited but, due to the high mortality factor associated with patients receiving blood products, it is estimated that the transfusion of virally inactivated FFP, instead of a standard pack, prolongs survival by just one hour and 11 minutes.[22] However, this slight benefit is probably negated by the hazards associated with the inactivation process.

Obviously, there are more health benefits to be gained from pathogen inactivation of platelets, especially if the need to irradiate for selected patients at risk from TA-GVHD is removed, and the problems due to bacterial contamination are eliminated. Currently, cost effectiveness must be considered for each product as each is processed differently. The ideal approach would be the use of one all-embracing technique on the complete donation before it is split into components. The development of new techniques using riboflavins may offer pathogen inactivation techniques appropriate to FFP, red cells and platelet concentrates.[24,25]

Whichever inactivation technology is chosen, its introduction will have implications for NBS personnel, and this ongoing issue is likely to result in changes as research is put into practice. It has been suggested that it may not be necessary to employ sophisticated screening tests in tandem with pathogen inactivation processes, and some savings could be made by discontinuing less-informative screening tests.[26]

Government legal advice is that any patient who becomes infected following a transfusion may have the basis of a claim against the NBS if technology for pathogen inactivation is available but not used.[26]

Errors in the chain of events leading to a transfusion contribute significantly to adverse transfusion outcomes; however, the introduction of pathogen inactivation techniques will not lead to a reduction in such events. It has been suggested that strategies to improve the level of safety in this area would be far more cost-effective, and these should be considered.[27]

The current tendency towards the introduction of procedures thought to increase transfusion safety appears to be driven by public concern, media frenzy, politics and the fear of litigation. These may be the driving force behind the introduction of new techniques, rather than an evidence-based, cost-effective scientific decision, but such techniques may move zero-risk blood transfusion a step closer. □

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The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

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COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

NOTE FOR GUIDANCE ON ASSESSING THE RISK FOR VIRUS TRANSMISSION – NEW CHAPTER 6 OF THE NOTE FOR GUIDANCE ON PLASMA-DERIVED MEDICINAL PRODUCTS (CPMP/BWP/269/95)

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7 Westferry Circus, Canary Wharf, London, E14 4HB, UK
Tel. (44-20) 74 18 84 00 Fax (44-20) 74 18 85 45
E-mail: mail@emea.eu.int http://www.emea.eu.int

NOTE FOR GUIDANCE ON ASSESSING THE RISK FOR VIRUS TRANSMISSION – NEW CHAPTER 6 OF THE NOTE FOR GUIDANCE ON PLASMA-DERIVED MEDICINAL PRODUCTS (CPMP/BWP/269/95)

TABLE OF CONTENTS

1. INTRODUCTION

The aim of this chapter is to outline the general principles that manufacturers should follow in performing a risk assessment with respect to potential virus transmission from plasma-derived medicinal products and the basis for its evaluation by the competent authorities.

2. GENERAL PRINCIPLE OF THE RISK ASSESSMENT

The principle of the risk assessment is to weigh up the potential amount of a given virus that may be present in the starting material ("potential virus input") against the capacity of the manufacturing process to inactivate or remove the contaminant virus ("overall virus inactivation/removal capacity"). In addition, by considering the amount of starting material needed to manufacture a single dose of product, the probability of potential virus contamination in a single dose of the final product can be estimated. The purpose of this assessment is to evaluate whether there is an adequate safety margin.

There are various factors, such as epidemiology, viraemic titre, virus inactivation/removal steps and product yield, that influence the level of infectious virus particles in a dose of final product and the reliability of the risk assessment will depend on the extent of information available on these factors. Many of these factors may vary and worst case scenarios should be considered. Since the minimum infectious dose for humans for many viruses is not known and since the stability of virus-antibody complexes during the manufacturing steps is usually unclear, it should be assumed that each virus particle entering the plasma pool might be infectious.

2.1 Potential virus input

For viruses that are potential contaminants of human plasma, the amount of virus that may contaminate the plasma pool for manufacture ('potential virus input') should be estimated. The 'potential virus input' is determined by the number of viraemic donations that could enter the manufacturing pool, the volume of individual donations and the titre of a viraemic donation.

The number of viraemic donations depends on the epidemiology in the donor population and on the frequency of donations from an individual donor. Donor selection and exclusion criteria, as well as inventory hold measures, should be assessed for their effectiveness in decreasing the number of viraemic donations that may enter the manufacturing pool. Any available information on the specific donor population from the Plasma Master File should be incorporated into the risk assessment. In cases where such data are not available, information should be sought from other sources e.g. general epidemiological surveys or investigational studies on the donor population.

The viraemic period should be described with respect to its length and virus titre. Testing of single units is the most sensitive strategy to exclude viraemic donations. In cases where donors are individually screened by specific tests, the titre of viraemic donations that are not recognised by such tests (e.g. donations from the 'window period') has to be considered.

A 'minipool' represents a defined number of aliquots of donations that are pooled for testing purposes. Testing of minipools (e.g. by nucleic acid amplification technologies (NAT)) may be a valuable tool in identifying and excluding highly viraemic donations. If this strategy is applied, the titre of viraemic donations that will not be detected should be determined.

Sensitive assays such as NAT can define an upper limit for virus contamination of the plasma pool. However, the sensitivities of tests applied to the manufacturing pool are limited and measures that identify and exclude contaminated donations may also result in the viral input being below that which can be detected at the manufacturing pool level.

2.2 Overall virus inactivation/removal capacity

The principles for determination of the virus inactivating/removal capacity of a production process and for interpretation of these data have been outlined in the CPMP guideline on virus validation (CPMP/BWP/268/95). Virus validation is an approach that has to be interpreted carefully with respect to reliability of the data from scaled-down experiments using model viruses or specific laboratory strains. The reliability of the virus reduction factors with respect to variations of manufacturing process parameters should be carefully considered. Despite the care that has to be applied to virus

validation in order to generate reliable data, there may remain some limitations, for example, the validity of summing-up logarithmic reduction numbers from single steps should be considered carefully and justified. The relevance of the viruses used in validation studies (model viruses or specific laboratory strains from the same species) should also be discussed.

For emerging viruses, the specific physical characteristics of the emerging virus should be discussed carefully with respect to any model viruses for which data have previously been derived. If it is possible to handle the emerging virus in the laboratory, investigational studies are recommended to evaluate the relevance of previously derived data. If it is not possible to use the emerging virus for validation studies, the use of a closely related model virus should be considered if pre-existing data were derived using viral species that are not adequate models of the emerging virus. Depending on the available data, further validation with the relevant virus or a more specific model virus should be decided on a product-specific basis.

2.3 Contribution from specific antibodies to virus safety

Specific antibodies may contribute to virus safety. A specification of the antibody content in the final product and validation of its neutralisation capacity could substantiate the role of specific antibodies in assuring the virus safety of a specific product. However, the benefit of specific antibodies in the pool for fractionation is difficult to assess as there is no reliable information on viral neutralisation at this manufacturing stage nor on the stability of virus-antibody complexes during further downstream processing.

2.4 Estimation of virus particles per dose

The amount of plasma used for production of one dose of final product should be defined considering the product yield from plasma, the batch sizes, the number of vials produced from a batch and the number of vials per treatment (dose). The relevant data should be provided from process validation. Comment should also be made on the use of the product, e.g. single use versus continuous treatment.

The potential number of virus particles per dose of product can be calculated from the viral titre in the starting material, the volume of plasma required to produce one dose and the viral clearance factor obtained from validation studies. An example of such a calculation is outlined in Appendix 5 of the ICH guideline on viral safety (CPMP/ICH/295/95). As a general principle, for a safe product, the virus inactivation/removal capacity should clearly exceed the potential amount of virus that could enter the production process, leading to an adequate safety margin for a dose of final product. The number of estimated virus particles per dose may be discussed in respect to what is known about the minimum human infectious dose. Any statement about the human infectious dose included would have to be substantiated by valid data regarding the route of administration. If such data are not available, a conservative approach using viral genomes as an indicator of potentially infectious virus particles in the starting material should be followed.

3. CLINICAL EXPERIENCE AND SURVEILLANCE

The clinical experience with respect to virus transmission from the product, including any reports of virus transmission with the product or any similar product, should be discussed. However, an absence of reported transmissions does not prove the viral safety of a product in each case because undetected transmissions may have occurred or the product may have been used in a non-susceptible population. Further, the number of investigated patients from clinical studies is usually too low to detect infections, and experience and pharmacovigilance cannot be applied to emerging viruses or viruses that are not detected or carefully considered by this system.

4. APPLICATION OF THIS GUIDELINE

A viral risk assessment for HIV, HBV, HCV, B19 and HAV should be performed for all new marketing applications. This will allow assessors to weigh up viral safety and will substantiate statements on virus safety and any remaining potential risk in the SPC, as outlined in the Note for Guidance on the Warning on transmissible agents in SPCs and Package Leaflets for plasma-derived medicinal products (CPMP/BPWG/BWP/561/03).

For products for which a marketing authorisation has already been obtained, a risk assessment will be expected for HAV and B19 if claims are made regarding effective measures for these viruses. If no claims are made, no risk assessment is required. In either case, risk assessments for HIV, HBV and HCV are not required.

A risk assessment will not be expected for any albumins manufactured by an established process where a general statement on virus safety is foreseen in the core SPC.

A risk assessment should be performed whenever post-pooling information indicates that a contaminated donation has entered the manufacturing plasma pool[1].

For emerging viruses, an appropriate risk assessment should be made with reference to any available position statements (e.g. for West Nile virus).

[1] Further guidance on the actions to be taken in this situation is provided in this Note for Guidance on Plasma-derived medicinal products in the section on source materials, and in Annex 14 to the EU guide to Good Manufacturing Practice.

The European Agency for the Evaluation of Medicinal Products
Human Medicines Evaluation Unit

London, 14 February, 1996
CPMP/BWP/268/95

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

NOTE FOR GUIDANCE ON VIRUS VALIDATION STUDIES: THE DESIGN, CONTRIBUTION AND INTERPRETATION OF STUDIES VALIDATING THE INACTIVATION AND REMOVAL OF VIRUSES

Revised *

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7 Westferry Circus, Canary Wharf, London E14 4HB, UK
Switchboard: (+44-171) 418 8400 Fax: (+44-171) 418 8551
E_Mail: mail@emea.eudra.org http://www.eudra.org/emea.html

VIRUS VALIDATION STUDIES: THE DESIGN, CONTRIBUTION AND INTERPRETATION OF STUDIES VALIDATING THE INACTIVATION AND REMOVAL OF VIRUSES

Note for Guidance

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CONTENTS

1. INTRODUCTION

1.1 This guideline discusses the need for and the contribution of viral validation studies towards the viral safety of biological products. The principal aims of the guideline are to provide guidance on the design of a validation study including the choice of viruses to be used and on the interpretation of the ensuing data especially with respect to defining a process step which can be considered to be effective in the inactivation and/or removal of viruses.

1.2 The guideline concerns the validation of virus inactivation and/or removal procedures for all categories of medicinal biological products for human use with the exception of live viral vaccines including genetically engineered live vectors. The type of products covered include:

- products derived from *in vitro* culture of cell lines of human or animal origin,
- products derived from in vivo culture of cell lines, or from organs or tissues of human or animal origin,
- products derived from blood or urine or other biological fluids of human or animal origin.

1.3 The risk of viral contamination is a feature common to all biologicals whose production involves the use of material of animal or human origin. Viral contamination of a biological may arise from the source material, e.g. cell banks of animal origin, human blood, human or animal tissues, or as adventitious agents introduced by the production process, e.g. the use of animal sera in cell culture.

1.4 In the past, a number of biologicals administered to humans have been contaminated with viruses. In several instances, the virus was only identified many years after the product had been introduced into the market since contamination occurred prior to adequate knowledge concerning the presence of the infectious agents. The primary cause of these viral transmissions has been contamination of the starting or source materials. Examples include Yellow Fever vaccine which was contaminated by avian leukosis virus by virtue of its production in naturally infected hens eggs, whilst SV40 was a contaminant of poliovirus and adenovirus vaccines prepared in the 1950's on primary cultures of kidney cells obtained from Rhesus monkeys naturally harbouring a clinically inapparent infection with SV40. In addition, viruses present in human plasma, e.g., HIV and HCV, have contaminated blood products whilst human growth hormone extracted from the pituitaries of cadavers has been implicated in the transmission of the aetiological agent responsible for Creutzfeldt-Jakob disease. Contamination of a biological can also arise from the use of infected material during production or as an excipient. Perhaps the most notable was Yellow Fever vaccine contaminated with HBV present in human serum used as a stabiliser in the 1940's.

1.5 Three principal complementary approaches can be adopted to control potential viral contamination of biologicals:

(i) selecting and testing source material for the absence of detectable viruses,

(ii) testing the capacity of the production processes to remove or inactivate viruses,

(iii) testing the product at appropriate stages of production for freedom from detectable viruses.

No approach provides a sufficient level of assurance alone and this will only be achieved using a combination of the above.

1.6 Testing of starting materials is essential to minimise viral contamination. While tests may be able to detect one or more virus species, no single test will be able to demonstrate the presence of all known viruses. Moreover all test systems require a minimum level of viral contamination to record a positive and tests are also limited by statistical considerations in sampling. Some tests, e.g. the test for antibody to HCV in human plasma, may measure markers of infection which only become positive sometime after infection. Similar considerations apply to testing of the final product.

1.7 Therefore establishing the freedom of a biological from infectious virus will in many instances not derive solely from direct testing for their presence, but also from a demonstration that the manufacturing process is capable of removing or inactivating them. Validation of the process for viral inactivation/removal can play an essential and important role in establishing the safety of biological products especially when there is a high potential for the source material to be contaminated with a virus known to be pathogenic for man, eg. plasma derived products. Also, since many instances of contamination in the past have occurred with agents whose presence was not known or even suspected at the time of manufacture, an evaluation of the process can provide a measure of confidence that a wide range of viruses including unknown, harmful viruses, may be eliminated.

1.8 The intention of this note for guidance is to provide a general framework for validation studies and the virological approach which should be used in the design of virus validation studies. Manufacturers should apply the recommendations presented here to their specific product taking into consideration the nature of the source material, the procedures used for production and purification and any other factors which can have consequences on this safety issue. The approach used by manufacturers in studies for evaluating virus elimination should be explained and justified.

2. SOURCES OF VIRAL CONTAMINATION

Viral contamination of biologicals can arise in the following ways:

2.1 Source material may be contaminated with a virus indigenous to the species of origin. Blood can harbour many viruses and the use of products derived from human plasma has caused infections by HBV, HCV, HIV, parvovirus B19 and occasionally HAV. Murine viruses, some of which are pathogenic for man, may contaminate murine hybridomas. Cell lines which are intended to be used for genetic manipulation may be contaminated by viruses and, therefore, they should be chosen carefully and tested for freedom from detectable adventitious agents even before genetic manipulation, in order to start with a well characterised cell line.

2.2 Cells may have a latent or persistent infection, for example, a herpes virus or a retrovirus, which may be transmitted vertically from one cell generation to the next as a viral genome and which may be expressed intermittently as infectious virus.

2.3 The process of construction of a production cell line may introduce a contaminant virus indigenous to another species, e.g. an EBV transformed human lymphoblastoid cell line secreting a monoclonal antibody can be infected with a murine retrovirus after fusion with a murine myeloma.

2.4 Adventitious viruses may be introduced by the use of contaminated animal products in the production process e.g. cell cultures may be contaminated with bovine viruses through the use of bovine sera or a murine monoclonal antibody used in affinity chromatography may contaminate a product with a murine virus.

2.5 Other sources of contamination, eg., operating personnel or raw materials of non-biological origin, are possible.

3. THE VALIDATION PROCESS

3.1 The aim of viral validation studies is:

(i) to provide evidence that the production process will effectively inactivate/remove viruses which are either known to contaminate the starting materials, or which could conceivably do so, and

(ii) to provide indirect evidence that the production process might inactivate/remove novel or unpredictable virus contamination.

This is achieved by deliberately adding ('spiking') a virus to material at various production steps and measuring its removal or inactivation during the subsequent individual step or steps. This will identify production steps which are effective in reducing the level of infectious virus and provide an estimate of the overall ability of the process to eliminate contaminating viral infectivity.

3.2 Virus validation studies, as with direct testing of materials at appropriate steps, contribute to confidence in the virological safety of the product. However, all virus validation studies must be regarded as an approximation to the true capacity of the process since it may be difficult or impossible to conduct a perfect validation study of a process because of the large numbers of complex variables involved. Results have shown that even small modifications in procedure or the particular laboratory strain of virus used can have a large effect on virus removal or inactivation.

3.3 Where the starting or source material is less well characterised, such as blood, tissues and organs of human or animal origin, or when cells have been cultured by *in vivo* techniques, there is a higher possibility of viral contamination and the manufacturing process will normally incorporate one or more effective virus inactivation/removal steps. Products derived from human plasma raise particular viral safety concerns and specific guidance is given in the CPMP guideline on 'Medicinal Products Derived From Human Plasma (Revised)'.

3.4 In the past, where the starting material posed a lower virological risk, such as a fully characterized cell bank, the purification process often did not contain a specific virus inactivation/removal step and a validated purification process was considered to give sufficient levels of viral inactivation/removal. Clinical experience has not revealed any problems with this approach. However, some manufacturers of monoclonal antibodies (mAbs) are introducing specific viral inactivation/removal steps into their production process since mAb producing cell lines of murine origin inevitably secrete variable quantities of retroviruses which may be infectious.

3.5 It should be borne in mind that cell culture systems inherently support virus replication. Therefore, a distinct low level of risk of viral contamination of the culture persists despite a high level of cell bank characterization and occasional cases of adventitious virus contamination have been reported.

3.6 The justification for, and the extent of, the required validation studies will vary depending on the manufacturing process and type of product (eg., species of origin of starting material, whether source material is variable or defined, stability of the active material, etc.). The appropriateness of the studies will be reviewed on a case-by-case basis.

4. THE CHOICE OF VIRUSES FOR VALIDATION

4.1 Viruses for validation should be chosen firstly to resemble viruses which may contaminate the product as closely as possible and secondly to represent as wide a range of physico-chemical properties as possible in order to test the ability of the system to eliminate viruses in general.

4.2 Most validation studies employ laboratory strains of virus which can be produced and assayed conveniently. However, experience has shown, and manufacturers should be aware, that different laboratory strains of virus may have different properties from each other and from naturally occurring viruses. Consequently, any virus used in a validation study is actually a model virus. The manufacturer should justify the choice of viruses in accordance with the aims of the validation study and the principles laid down in this guideline. Unless otherwise justified, where two similar viruses could be used for validation studies either because of their equal resemblance to possible contaminants or similarities in their properties, the virus considered to be the more resistant should be used.

4.3 Examples of the choice of viruses are:

(i) Human plasma-derived clotting factor concentrates have been contaminated by HIV. Thus the production process for such materials must be evaluated for its ability to inactivate/remove infectious HIV.

(ii) Cell lines derived from rodents usually contain endogenous retroviral particles which may be infectious (C-type particles) or non-infectious (A-type particles). Where the source material is obtained from rodent cell lines, the production process should be evaluated for its ability to inactivate/remove one of the closely related laboratory murine retroviruses.

(iii) Examples of viruses representing a range of physico-chemical properties which have been used to evaluate the general ability of a process to remove virus infectivity include:

a) SV40, poliovirus or an animal parvovirus as small non-enveloped viruses,

b) a parainfluenza or a murine retrovirus as large enveloped RNA viruses,

c) a herpesvirus as a large DNA virus.

Examples of viruses which have been used in the past in validation studies are given in Table 1. However, since these and the viruses mentioned above are merely examples, the use of any of them is not mandatory and manufacturers are invited to consider other viruses especially those which may be more appropriate for their individual processes. Further guidance on the choice of viruses for the validation of manufacturing processes of plasma derivatives is provided in the CPMP guideline 'Medicinal Products Derived From Human Plasma (Revised)'.

4.4 There should be an efficient, sensitive and reliable infectivity assay for the viruses used. Viruses which can be grown to high titre will be desirable, although this may not always be possible.

4.5 Products derived from ovine, caprine or bovine tissues raise the problem of contamination by agents of transmissible spongiform encephalopathy, such as scrapie, which accumulate in the central nervous system and lymphoid tissue. These agents are the subject of a separate note for guidance (Note for guidance for minimising the risk of transmitting agents causing spongiform encephalopathy via medicinal products. III/3208/91-EN).

5. DESIGN OF VALIDATION STUDIES

5.1 Validation studies involve the deliberate addition of a virus at various production steps and measuring the extent of its removal/inactivation during the subsequent individual step or steps. It is not necessary to validate every individual step of a manufacturing process. Only those steps which are likely to contribute to inactivation/removal of a virus need to be subject to a validation study.

5.2 GMP restraints prevent the deliberate introduction of any virus into the production facilities. Therefore, the validation should be conducted in a separate laboratory equipped for virological work on a scaled-down version of the production process and performed by staff with virological expertise in conjunction with the production bioengineers. Studies should be carried out in accordance with the principles of GLP.

5.3 The comparability of the model and full scale procedures is the premise on which the results obtained with the scaled-down system can be accepted in evaluating the virus safety of the product. Therefore, the validity of the scaling down should be demonstrated, by comparison of process parameters such as pH, temperature, concentration of protein and other components, reaction time, column bed height, linear flow rate, flow rate to bed height ratio, elution profile and step efficiency (eg., yield, balance, specific activity, composition). Deviations which cannot be avoided should be discussed with regard to any potential influence on the results.

5.4 Whenever possible, it should be shown whether the reduction in virus infectivity is accomplished by inactivation of virus or by removal of virus particles. This may be achieved by establishing the kinetics of loss and/or a balance of infectivity, as appropriate. Processes which reduce virus infectivity by inactivation are potentially more easily modelled than those which physically remove particles. For a viral inactivation step, the kinetics of inactivation should be studied and included in both tabular and graphical form in reports. Where the inactivation is too rapid to plot the kinetics using process conditions, further studies should be performed in order to prove that infectivity is indeed lost by inactivation. Thus appropriate controls should be introduced to detect possible interference with the assay from the sample or the matrix into which it is introduced and the limits of detection should be established.

5.5 Production parameters which influence the effectiveness of a process step to inactivate/remove viruses should be explored and the results used in setting appropriate in-process limits. Critical parameters include:

- mechanical parameters such as flow rates, mixing rates, column dimensions, column reuse, etc.
- physicochemical parameters such as protein content, pH, temperature, moisture content, etc.

5.6 Antibodies present in the starting material may affect the behaviour of a virus in partition or inactivation steps. Validation studies should take this into account.

5.7 The validity of the log reduction achieved will be established from investigation of the effects of variation in critical process parameters used to set in-process limits.

5.8 Published work concerning the ability of related or generic processes to inactivate/remove viruses may provide an indication of which steps are likely to be effective. However, the variability intrinsic to validation studies arising from the need to model the process, choose viruses to be used and explore full scale production parameters on a laboratory scale, means that validation data must be based on experimental studies provided by the

5.9 The amount of virus added to the starting material for the production step which is to be studied should be as high as possible in order to determine the capacity of the production step to inactivate/remove viruses adequately. However, the virus spike should be added such that the composition of the production material is not significantly altered (typically the volume of the virus spike will be less than 10%). Whenever possible, calculated reduction factors should be based on the virus which can be detected in the spiked starting material and not on the amount of virus added.

5.10 If possible, virus in samples from model experiments should be titrated without further manipulations such as ultra-centrifugation, dialysis or storage. Where further treatments are unavoidable, e.g. to remove inhibitors or toxic substances, or storage for a period to ensure that all samples are titrated together, appropriate controls should be included to determine what effect the procedures have on the result of the study. Effects of the sample on the detection system, including toxic effects, should be recorded as they influence the limits of detection.

5.11 Quantitative infectivity assays should be performed according to the principles of GLP and may involve plaque formation, detection of other cytopathic effects such as syncytia or foci formation, end point titrations (eg., $TCID_{50}$ assays), detection of virus antigen synthesis or other methods. The method should have adequate sensitivity and reproducibility and should be performed with sufficient replicates and controls to ensure adequate statistical accuracy of the result (see Appendix I).

5.12 Nucleic acid amplification methods, e.g., PCR, are a promising approach capable of great sensitivity in detecting viral genomes and also can detect viruses such as hepatitis B and C for which culture systems are not available. However, an important limitation of the technology is that inactivated virus may still score positive in a genome amplification assay and thus may underestimate the degree of virus inactivation obtained by a potentially effective step. On the other hand, PCR may be of value in studies of processes which depend on virus removal. The use of this technology poses major problems in terms of quantification, standardisation, quality control and interpretation of results. Validation and standardisation of these assays must be unambiguously demonstrated before they are acceptable and extreme caution used in interpretation of both positive and negative results.

5.13 Assurance should be provided that any virus potentially retained by the production system will be adequately destroyed prior to reuse of the system, e.g. by sanitization of columns, etc.

6. INTERPRETATION OF DATA

6.1 A combination of factors must be considered when judging the effectiveness of a virus inactivation/removal step. Assessment of a step based solely on the quantity of virus inactivated/removed can lead to the conclusion that a process meeting specified levels of virus reduction will produce a safe product. This is not necessarily the case. The following factors all contribute in defining the effectiveness of a step and the data must be carefully evaluated in each case:

(i) The appropriateness of the test viruses used (see Section 4).

(ii) The design of the validation studies (see Section 5).

(iii) The $\log_{10}$ reduction achieved. Log reductions of the order of 4 logs or more are indicative of a clear effect with the particular test virus under investigation. However, it is emphasised that log number reduction cannot be used as the single, absolute measure of the effectiveness of a step.

(iv) The kinetics of inactivation. This will indicate whether or not the measured log reduction is a conservative estimate. Virus inactivation is usually not a simple first order reaction and often has a fast initial phase followed by a slower phase. However, a dramatic reduction in the rate of inactivation with time may suggest a loss of effectiveness of the inactivating agent or that a residual virus fraction is resistant to the inactivating agent and implies that the step is neither highly effective nor robust.

(v) The nature of inactivation/removal and whether it is selective for only certain classes of virus. A process step may be highly effective for some viruses but ineffective against others, eg., S/D treatment is effective against lipid-containing but not lipid-free viruses.

(vi) The susceptibility of virus inactivation/removal to small variations in process parameters will affect the confidence placed in a step.

(vii) The limits of assay sensitivities.

It is the combined evaluation of the above factors that will lead to a decision on whether a process step can be regarded as effective, moderately effective or ineffective in the inactivation/removal of viruses.

6.2 The following examples are intended to illustrate some of these principles and are neither definitive nor all encompassing:

(i) Where a process step is challenged with 6 logs of virus and 4 logs are recovered, the step cannot be claimed to be effective, although it may contribute to overall removal.

(ii) Where a process step is challenged with 6 logs of virus, but because of the cytotoxicity of the product the limit of assay sensitivity in the product is 4 logs, only 2 logs of removal have been demonstrated, and the step cannot be claimed to be effective. The process step may in fact be able to remove far greater quantities of virus, which might be demonstrated by a different experimental design.

(iii) Where a process step is challenged with 6 logs of virus and 2 logs are recovered, substantial amounts of virus have been removed. The product is not virologically sterile. However, if this reduction is reproducible and not influenced by process variables, the step is of some efficacy. It contributes to overall reduction of virus load and may be counted as such.

(iv) Where a process step is challenged with 6 logs of virus and no virus is detected in the product with a limit of sensitivity of the order of 2 logs, approximately 4 logs of removal have been demonstrated. This is substantial and the step may in fact remove far greater quantities than can be quantified or claimed.

(v) Where virus is inactivated, the kinetics of loss of infectivity are important. If a process step involves prolonged incubation, e.g. heating for ten hours, and infectivity reaches the limits of detection rapidly, the process is likely to have a greater virucidal effect than can often be demonstrated. On the other hand, if infectivity is lost slowly and the limits of detection are reached towards the end of the treatment period, the step provides less assurance of viral safety.

6.3 In general, partition processes are not considered to be effective viral removal steps although it is recognised that they can contribute to virus removal. Partition processes usually have a number of variables that are difficult to control and are often difficult to scale down for validation purposes. Furthermore, partitioning is dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties. Thus a model virus may be partitioned in a completely different manner to a target virus because of relatively minor differences in surface properties such as glycosylation. Even a relevant virus propagated in the laboratory may act differently from the wild-type virus in this respect. However, if a partition process gives reproducible reduction of virus load and if manufacturing parameters influencing the partition can be properly defined and controlled and if the desired fraction can be reliably separated from the putative virus-containing fraction, then it could fit the criteria of an effective step.

6.4 The objective of the validation is to identify steps effective in the inactivation/removal of viruses and to obtain an estimate of the overall capacity of the manufacturing process to inactivate/remove them. An overall reduction factor is generally expressed as the sum of individual factors (see Appendix II). However, a simple summing of low individual reduction factors may be misleading. Reductions in virus titre of the order of 1 log or less are considered to be unreliable because of the limitations of virus validation studies and should be ignored. Manufacturers should differentiate effective steps from process steps which may contribute to removal but upon which less reliance can be placed. Consideration should also be given to whether virus surviving one step would be resistant to a subsequent step or alternatively have increased susceptibility. In general, a single step having a large effect gives more assurance of viral safety than several steps having the same overall effect.

6.5 If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced.

6.6 For all viruses, manufacturers will be expected to justify the acceptability of the reduction factors obtained. Results will be considered on a case-by-case basis.

6.7 The GMP principle that material subjected to an effective virus inactivation/removal step should be segregated from untreated material should be rigorously applied.

7. LIMITATIONS OF VALIDATION STUDIES

Validation studies are useful in contributing to the assurance that an acceptable level of safety in the final product is established and do not by themselves establish safety. A number of factors in the design and execution of virus validation experiments may lead to an incorrect estimate of the ability of the process to remove naturally occurring virus infectivity. These factors include the following points.

7.1 Experience has shown that different laboratory strains of virus may differ in their sensitivity to the same treatment. The particular virus chosen may therefore not resemble the virus for which it has been chosen as a model. Native viruses may have unpredicted properties, for example association with lipid, which may affect their

properties.Virus preparations used to validate a production process are likely to be produced in tissue culture. The behaviour of tissue culture virus in a production step may be different from that of the native virus for example if native and cultured viruses differ in purity or degree of aggregation. The strains of virus, their cultivation and assay, and details of sampling and storage should all be documented.

7.2 There are some situations in which it may not be valid to add logarithmic reductions. For example, if a matrix is able to adsorb 10^4 infectious units of a virus and then cannot adsorb further material with comparable affinity then it will remove all virus when challenged with 10^4 infectious units, but only 1% when challenged with 10^6. The clearance measured may therefore differ with the challenge titre.

7.3 Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. As a consequence, the overall reduction factor is not necessarily the sum of reduction factors calculated from each individual step in which a fresh virus spike suspension is used. For example if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

7.4 Model scale processing is likely to differ from full scale processing despite care taken to design the scaled down process.

7.5 The presence of antibodies to a native virus may affect partition of the virus or its susceptibility to chemical inactivation; but it may also complicate the design of the study by neutralising infectivity. The appropriateness of the study design may be difficult to judge. The level of antibody present may be considered a significant process variable.

7.6 Small differences in production parameters such as protein content or temperature can produce large differences in the reduction of virus infectivity by whatever mechanism.

8. RE-EVALUATION STUDIES

8.1 Changes to the production process may necessitate a new validation study.

8.2 As scientific experience accumulates, processes will require re-examination to ensure that they remain of an acceptable standard.

APPENDIX 1

STATISTICAL EVALUATION OF VIRUS TITRES AND REDUCTION FACTORS AND ASSESSMENT OF THEIR VALIDITY

Virus titrations suffer the problems of variation in common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays are therefore necessary to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue culture infectious dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titres are then measured by the proportion of animals or cultures infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between assay variation) than when results within a single assay run are compared (within assay variation).

3. The 95% confidence limits for within assay variation and for between assay variation normally should be of the order ± 0.5 $\log_{10}$ or better. Between assay variation can be monitored by the inclusion of a in-house reference preparation, the estimate of whose potency should be within approximately 0.5 $\log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Within assay variation can be assessed by standard textbook methods. In any particular experiment, if the precision of the titration is less than these target figures, the study may still be acceptable if justified.

4. The reduction in virus load should be calculated from the experimentally determined virus titres. The 95% confidence limits of the reduction factors should be obtained wherever possible. They can be approximated by $\pm\sqrt{(s^2 + a^2)}$, where $\pm s$ is the 95% confidence limits for the viral assays of the starting material, and $\pm a$ for the viral assays of the material after the step.

If after an inactivation/removal step no sample shows signs of infectivity, a reduction factor cannot be estimated by statistical means. To obtain an estimate of a minimum reduction factor, the titre should be expressed as less than or equal to one infectious unit in the volume of the highest concentration tested. Especially after potent inactivation processes, it can be expected that no sample shows signs of infectivity. To make the estimated minimum reduction factor of an effective inactivation process as large as possible, as much processed undiluted material as possible should be sampled.

APPENDIX II

CALCULATION OF REDUCTION FACTORS

The virus reduction factor, R, for an individual inactivation or removal step is given by the expression:

$$R = \log \frac{V1 \times T1}{V2 \times T2}$$

where, R = the reduction factor,

V1 = volume of starting material,

T1 = concentration of virus in starting material,

V2 = volume of material after the step, and

T2 = concentration of virus after the step.

This formula takes into account both the titre and the volume of the material before and after the step.

Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. The European Pharmacopoeial convention[1] with respect to methods of sterilisation is that processes which deliver a sterility assurance level (SAL) of 10^{-6} or better for bacteria, moulds and yeasts are considered adequate. A SAL of 10^{-6} denotes a probability of not more than one viable micro-organism in 1×10^{-6} sterilised items of the final product.

[1] "Methods of Preparation of Sterile Products" monograph of the European Pharmacopoeia

Table 1 **EXAMPLES OF VIRUSES WHICH HAVE BEEN USED IN VIRUS VALIDATION STUDIES**

Virus	Family	Genus	Natural Host	Genome	Env	Size	Shape	Resistance to Physicochemical Treatment*
Vesicular stomatitis virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	Yes	70x175 nm	Bullet shaped	Low
Parainfluenza virus	Paramyxo	Paramyxovirus	Various	RNA	Yes	100-200nm	Pleo/Spher	Low
Human immuno-deficiency virus	Retro	Lentivirus	Man	RNA	Yes	80-100nm	Spherical	Low
Murine leukaemia virus (MuLV)	Retro	Type C oncovirus	Mouse	RNA	Yes	80-110nm	Spherical	Low
Sindbis virus	Toga	Alphavirus	Man?	RNA	Yes	60- 70nm	Spherical	Low
Bovine viral diarrhoeal virus (BVDV)	Toga	Pestivirus	Bovine	RNA	Yes	50-70nm	Pleo-Spher	Low
Pseudorabies virus	Herpes	Varicellovirinae	Swine	DNA	Yes	120-200nm	Spherical	Med
Poliovirus, Sabin type 1	Picorna	Enterovirus	Man	RNA	No	25-30nm	Icosahedral	Med
Encephalomyocarditis virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	No	25-30nm	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	No	60-80nm	Spherical	Med
Hepatitis A	Picorna	Hepatovirus	Man	RNA	No	25-30nm	Icosahedral	High
SV40	Papova	Polyomavirus	Monkey	DNA	No	40-50nm	Icosahedral	V.High
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	No	18-24nm	Icosahedral	V.High

This Table gives an incomplete list of viruses which have been used in validation studies. Consequently, the use of any of the viruses in the Table is not mandatory and manufacturers are invited to consider other viruses especially those which may be more appropriate for their individual production processes.

Information Sheet

Ensuring the Quality and Safety of Plasma Derived Medicinal Products

Scope

This information sheet is intended to provide advise to National Medicine Regulatory Authorities, blood transfusion services, policy makers in national health authorities, manufacturers of plasma derived medicinal products and interested parties on the fundamental aspects supporting the quality and safety of these products.

Plasma Derived Medicinal Products

Plasma derived medicinal products are manufactured from human blood plasma (*plasma*). Plasma can be obtained from whole blood donations (*recovered plasma*) or by apheresis procedures (*source plasma*). Plasma is the source of a wide range of medicinal therapeutic products that are used for the treatment and prevention of a variety of life-threatening injuries and diseases often associated with protein deficiency states.

Improvements in protein purification technology (*fractionation*) over recent years have made available a wide variety of human plasma proteins that include:

- Coagulation factors
- Immunoglobulins
- Albumin
- Fibrin sealants

The transmission of blood-borne pathogens, such as hepatitis and Human Immunodeficiency Virus (HIV) are of particular concern in the manufacture of plasma derived medicinal products. A batch of starting plasma containing a single contaminated unit of plasma potentially can transmit a blood-borne disease to a large number of recipients.

From the contaminated plasma pool multiple intermediate products and subsequently numerous batches of final product can be manufactured. Therefore, the safety of products manufactured from plasma, is dependent on the measures taken to minimise the contamination of the starting material (donor selection, screening and testing). Safety is enhanced by the application of virus inactivation procedures and technology that removes or reduces the level of blood-borne viruses and other infectious agents.

The national Medicines Regulatory Authorities (MRAs) are responsible for the regulation of plasma derived medicinal products. Over the past decades, they have been faced with serious and complex challenges at a scientific, technological and regulatory level to ensure that these biological products are of good quality, safety and efficacy.

Quality Assurance Principles

Conventional pharmaceutical products tend to be produced from chemically defined raw materials and the manufacture

is controlled using reproducible chemical and physical techniques. Plasma derived medicinal products are inherently variable due to their biological nature, and the biological methods used to test them. Because of the complexity and variability, a high level of expertise is required for the regulation and batch release of these products. As with other biologicals, four principal complementary approaches should be adopted to assure quality and safety:

- *Starting Material:* Assurance of the quality and safety of the plasma for fractionation
- *Manufacturing technique:* Control of fractionation and subsequent manufacturing procedures for isolation, purification, virus removal and inactivation
- *Good Manufacturing Practice (GMP):* Strict adherence to GMP and the prevention of cross contamination
- *Product Compliance:* Standardisation of biological methods needed in the characterisation of in-process and final products.

National Medicines Regulatory Authorities: Standards setting

National MRAs have the duty to ensure that the available pharmaceutical products, whether imported or manufactured locally, are of high quality, safe and efficacious. Plasma derived medicinal products should be included within the legal definition of pharmaceutical products, and fall under the jurisdiction of national MRAs. This function should have a firm statutory and legislative framework.

A national MRA should be an entity fully independent from the manufacturer, undertaking its responsibility in an independent, legal, authoritative and impartial manner.

National Medicines Regulatory Authorities: Responsibilities

Regulations

Individual Member States should develop and institute appropriate national regulations for plasma derived medicinal products. The regulations should take into account specific products, general products, starting materials and be authorised in the respective countries.

They should be based on current international standards, such as those available from WHO and other regulatory authorities. National MRAs should actively participate in initiatives towards international harmonisation of regulation.

Documentation

The national MRA should develop documentation to facilitate the application for registration (market authorisation, licensing) of plasma derived medicinal products.

Facility Documentation

As part of the licensing procedure, details of the manufacturing buildings the location, construction, service facilities and details of the environment should be consolidated in a file (*Site Master File).*

Plasma Documentation

As part of the licensing procedure, for plasma derived medicinal products, information on the collection and control of the starting plasma material, should be documented (*Plasma Master File).*

Such a system aims to ensure quality as well as the traceability of each plasma unit from the donor, through the manufacturing process to the recipient of the product and vice-versa. Documentation should record such information as epidemiological data, the tests performed on the donor, donation, plasma unit, the

plasma pool, and post-pooling information on the manufacture and control of the starting material.

National MRA are responsible for approving the appropriate selection, use and validation of the types of diagnostic tests and test kits used for assaying viral markers. These are used in the testing of blood donations, plasma units and plasma pools prior to entering the fractionation process.

Inspection
The national MRA has the responsibility to confirm that the manufacturer is adhering to the approved standards of good manufacturing practice and to national and other requirements for the manufacture and quality control of specific products.

Good Manufacturing Practices (GMPs)
The manufacture of plasma products should be undertaken in accordance with the principles of good manufacturing practices (GMP).
All the steps in their manufacture affect the quality of the final products, therefore all operations should be done in accordance with an appropriate system of quality assurance and current GMP.

Batch Release
A batch of final product should be homogeneous. Product can be processed in a single process or combine a series of processes.
At the time the product is licensed, the national MRA should decide what level of control is going to apply to demonstrate the consistency of manufacture. The nature of the product and the history of the manufacturer may influence the MRA decision.

Licensing/Marketing Approval
The national MRA should only issue a license (registration, marketing authorisation) for a specified plasma derived medicinal product when it is satisfied that the product conforms to the applicable national and/or international requirements, the manufacturer's specifications and has successfully complied with a GMP inspection.

National MRA responsibility includes the approval of viral inactivation and removal procedures applied to the manufacture of plasma derived medicinal products.

International standards
The measurement of biological activity in human blood plasma products should be directly referenced to the WHO International Standards, defining an internationally agreed unit for a specific activity. Where it exists, the International Unit forms the basis for the establishment of clinical dosing and licensing.

To strengthen harmonisation, the national MRAs should establish appropriate national (*secondary standard*) reference materials calibrated against international reference materials. These can be made available to manufacturers.

Post marketing surveillance
Countries should establish a national system for the post-marketing surveillance of biological products. Clinicians and other health workers and professionals should be strongly encouraged to report to manufacturers and national MRAs unexpected adverse events occurring after administration of plasma derived medicinal products.

Recall and revocation
National MRAs should have a system for enforcing the recall of batches, revoking approvals and communicating such decisions to the manufacturer, users and to the MRAs of any countries importing the product.

References

WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives. WHO Technical Report Series No. 840 (Annex 2)

WHO Guidelines for National Authorities on Quality Assurance for Biological Products WHO Technical Report Series No. 822 (Annex 2)

Regulation and licensing of biological products in countries with newly developing Regulatory Authorities. WHO Technical Report Series No. 858 (Annex 1)

Good Manufacturing Practices for pharmaceutical products. WHO Technical Report Series No. 823 (Annex 1)

Good Manufacturing Practices for biological products. WHO Technical Report Series No. 822 (Annex 1).

Note: the information in this Fact Sheet does not involve the preparation and scope of application of blood components derived from single donations or small pools, which are used for direct transfusion to patients.

World Health Organization
Quality and Safety of Blood Products and Related Substances
Department of Essential Health Technologies
20 Avenue Appia, 1211 Geneva 27, Switzerland
Fax: +41 22 791 4836
www.who.int/biologicals
www.who.int/eht